GENE EXPRESSION IN THE STALLION TESTES

A Dissertation

by

ANDY MICHAEL LAUGHLIN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2010

Major Subject: Physiology of Reproduction

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Approved by:

Co-Chairs of Committee,	David Forrest
	Nancy Ing
Committee Members,	Tom Welsh
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ABSTRACT

Gene Expression in the Stallion Testes. (May 2010)

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Dr. Nancy Ing

Understanding the genes that regulate spermatogenesis and steroidogenesis in the testis is critical for enhancement of stallion fertility. Stallion testicular samples were used to identify candidate genes by cDNA microarrays that simultaneously assessed expression levels of 9132 genes. First, gene expression was compared between light (spermatogenically active) and dark (spermatogenically inactive) testis tissue of 1.5-year-old horses (n = 3). Ninety-three genes were differentially expressed (35 light specific, 58 dark specific) in matched paired samples. Second, gene expression was compared between testicular tissue of two mature stallions, one with normal quality semen (fertile) and one with poor quality semen (subfertile). A total of 233 genes were differentially expressed (122 in fertile tissue, 111 in subfertile tissue). Of these, phosphodiesterase 3B (PDE3B), steroidogenic acute regulatory (StAR) protein, and outer dense fiber of sperm tails 2 (ODF2) mRNAs, were localized and quantified by *in situ* hybridization (ISH) in mature stallions and/or in unilateral cryptorchids. ISH revealed differences ($P < 0.05$) among mature stallions (n = 10) for PDE3B (localized to seminiferous tubules) and StAR protein (localized to interstitial spaces) mRNAs. A

positive correlation coefficient (r = .556, p = .025) was found between StAR protein mRNA and plasma concentration of testosterone. Additionally, both gene products were evaluated in 1-year-old (n = 3) and 3-year-old (n = 3) unilateral cryptorchid stallions. Expression of both PDE3B and StAR protein gene was significantly higher in mature, descended testes compared to mature, retained testes and the descended and retained testes of immature, cryptorchid stallions. StAR protein gene demonstrated significantly higher expression in immature retained testes compared to immature descended testes.

A precision-cut tissue slice (PCTS) *in vitro* culture system was evaluated as a potential tool to study equine testes function. Testes from immature stallions (n = 3) were cut into slices (mean slice weight = 13.85 ± 0.20 mg; mean slice thickness = 515.00 ± 2.33 µm) and exposed to medium containing ovine luteinizing hormone (oLH) at concentrations of 0, 5, 50 and 500 ng/ml for 6 h at 32°C. Medium content of testosterone and estradiol was increased 500% and 120%, respectively, by addition of oLH versus that observed for the testis tissue slices treated with 0 ng oLH (control). An oLH concentration-dependent increase in StAR protein mRNA in tissue slices was detected by *in situ* hybridization; whereas, differences for PDE3B and ODF2 mRNAs were not observed. Collectively, these results demonstrate that the stallion is an excellent model for studying male fertility due to the initiation of spermatogenesis, frequency of cryptorchidism, and routine castration providing useful tissue to use for studying gene expression.

DEDICATION

This work is dedicated to my family. First, to Gina, the one who taught me by example how to persevere and gave me the reasons to complete what I had started. Secondly, this is dedicated to my children, Tristin, Andrea, and Jaxon. I pray that the mental, physical, and emotional costs of this work will be a blessing to you now and in the future.

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Additionally, I owe a great deal of gratitude to Cindy Balog-Alvarez and Pat Chen for their patience and for showing me how to function in a laboratory. I would also like to thank Dr. Parrish for the use of his laboratory and for showing me how research can be enjoyable. Others who have helped with the completion of this work include Dr. Noah Heninger, Dr. Charles Love, Linda Love, and Dr. John Nelson. To each, I would like to say thank you for your instruction and technical assistance. Without each of you, this project could not have been completed. Lastly, I bow low and thank God who gave me the ability to reach this milestone in my life.

NOMENCLATURE

AAMP	angio-associated, migratory cell protein
ABCA3	ATP-binding cassette 3
ABCF2	ATP-binding cassette, sub-family F (GCN20), member 2
ABP	androgen binding hormone
AD	Alzheimer disease
AIM1	absent in melanoma 1
AKAP12	A kinase (PRKA) anchor protein 12
ANKRD36	ankyrin repeat domain 36
ANOVA	analysis of variance
AR	androgen receptor
ARG2	arginase, type II
ASS1	argininosuccinate synthetase 1
ATP1A1	ATPASE, Na+/K+ transporting, 2 polypeptide
ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, B1
BTBD9	BTB (POZ) domain containing 9
BUB1B	budding uninhibited by benzimidazoles 1 homolog beta
CAH	congenital lipoid adrenal hyperplasia
cAMP	cyclic 3’, 5’-monophosphate
CCK	cholecystokinin
CCNA1	cyclin A1
CD63	CD63 molecule

CDC2	cell division cycle 2
CDH3	cadherin 3, type 1 (placental)
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic 5’-guanosine monophosphate
CITED1	CBP/P300-interacting transactivator with Glu/Asp-rich carboxy-terminal
CKS2	CDC28 protein kinase 2
CLDN1	claudin 11
CLGN	calmegin
CLK1	CDC-like kinase 1
CMYA5	cardiomyopathy associated 5
CNN3	calponin 3, acidic
CNTN2	contactin 2
CPE	carboxypeptidase E
CRYM	crystalline, mu
CTNNAL1	catenin (cadherin-associated protein) alpha-like 1
CTNNB1	catenin (cadherin-associated protein) beta 1, 88kD
CV	coefficient of variation
Cy3	cyanine green fluorescent dye
Cy5	cyanine red fluorescent dye
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide
CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide
DHCR24	24-dehydrocholesterol reductase

DHT	dihydrotestosterone
DMEM	Dulbecco's modified eagle's medium
DOC1	down regulated in ovarian cancer 1
DSO	daily sperm output
DSP	daily sperm production
DSP1	desmoplakin
DYS	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)
DZIP1	DAZ interacting protein 1
E_1	estrone sulfate
E_2	estradiol 17-β
EEF1B2	eukaryotic translation elongation factor 1 β 2
ERKO	estrogen receptor knockout
FBLN2	fibulin 2
FBS	fetal bovine serum
FOXG1	forkhead box G
FOXO3	forkhead box O3
FSH	follicle stimulating hormone
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GIFT	gamete intrafallopian transfer
GK	glycerol kinase
GLG1	Golgi apparatus protein 1
GNG3	guanine nucleotide binding protein, δ3

GnRH	gonadotropin releasing hormone
GSTO1	glutathione-S-transferase Ω
H&E	hematoxylin and eosin
HAGHL	hydroxyacyl gluthathione hydrolase
hCG	human chorionic gonadotropin
HCl	hydrochloric acid
HLA-B	major histocompatability complex, class 1, B
HLA-C	major histocompatability complex, class 1, C
HLA-G	major histocompatability complex, class 1, G
HPT	hypothalamic-pituitary-testicular axis
HSD	honest significant difference
HTR4	5-hydroxytryptamine (serotonin) receptor 4
HUWE1	HECT and WWE domain containing 1
ICSI	intracytoplasmic sperm injection
IFI6	interferon, alpha inducible protein 6
IGF2	insulin-like growth factor 2
IGFBP7	insulin-like growth factor binding protein 7
ISH	*in situ* hybridization
IVF	*in vitro* fertilization
JSD	juvenile spermatogonial depleted
KIF11	kinesin family member 11
KO	knockout

KRT8	keratin 8
LAMB1	laminin, β1
LDH	lactate dehydrogenase
LH	luteinizing hormone
LRPAP1	low density lipoprotein receptor-related protein 1
MBD3	methyl-CPG binding domain protein 3
MBNL1	muscleblind (drosophila)-like
MEST	mesoderm specific transcript homolog
MNS	morphologically normal sperm
mRNA	messenger ribonucleic acid
MRPL	mitochondrial ribosomal protein L
MRPL13	mitochondrial ribosomal protein L13
MSH4	mutS homolog 4 (*E. coli*)
MY040	hypothetical brain protein MY040
ODF2	outer dense fiber of sperm tails 2
OGT	O-linked N-acetylglucosamine transferase
oLH	ovine luteinizing hormone
ORC6L	origin recognition complex, subunit 6 like
$P450_{ssc}$	cytochrome P450 cholesterol side-chain cleavage enzyme complex
PAWR	PRKC, apoptosis, WT1, regulator
PBS	phosphate-buffered saline
PBX1	pre-B-cell leukemia homeobox

PBXIP1	pre-B-cell leukemia homeobox interacting protein
PCR	polymerase chain reaction
PCTS	precision-cut tissue slice
PDE	phosphodiesterase
PDE3B	phosphodiesterase 3B
PEDF	platelet epithelium derived factor
PHKG2	phosphorylase kinase, γ2 (testis)
PIGK	phosphatidylinositol glycan, class K
PKP4	plakophilin 4
PLCD1	phospholipase C, δ 1
PLD2	phospholipase D2
PMS	progressively motile sperm
PRKD1	protein kinase D1
PRM1	protamine
PROC	coagulation factor C
PROS1	protein S α
PTMS	parathymosin-cell cycle regulated
PURB	purine-rich element binding protein B
RGD	exonuclease NEF sp
RIA	radioimmunoassay
RNASE9	ribonuclease, RNASE A family 9
ROCK1	rho-associated, coiled-coil containing protein kinase

RPL	ribosomal protein
RT	room temperature
SATB1	SATB1 homeobox 1
SC4MOL	sterol-C4-methyl oxidase-like
SCSA	sperm chromatin structure assay
SD	standard deviation
SELENBP1	selenium binding protein 1
SEM	standard error of the mean
SEPT8	septin 8
SERP1	stress-associated endoplasmic reticulum protein
SERPINA5	serpine peptidase inhibitor, clade A, 5
SERPINF1	serpine peptidase inhibitor, clade F, member 1
SERPING1	serpine peptidase inhibitor, clade G, 1
SGCG	sarcoglycan
SLC2A14	solute carrier family 2, member 4
SORT1	sortilin
SPCS1	signal peptidase complex subunit 1 homolog
SPTAN1	spectrin, alpha, non-erythrocytic 1
StAR	steroidogenic acute regulatory protein
STK24	serine/threonine kinase 24
STMN1	stathmin 1
TCF4	transcription factor 4

TERT	telomerase reverse transcriptase
TGF	transforming growth factor
TMEM194A	transmembrane protein 194A
TRIM9	tripartite motif-containing 9
TUBA4	tubulin, alpha 4A
TUNEL	TdT-mediated dUTP Nick end Labeling
VRK1	vaccinia related kinase 1
ZEB2	zinc finger E-box binding homeobox 2

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CHAPTER I

INTRODUCTION

Identifying the causative mechanisms responsible for male infertility are challenging due to the numerous processes that affect gamete production such as spermatogenesis and steroidogenesis (Roser, 2008). Assisted reproductive technologies, such as gamete intrafallopian transfer (GIFT) and intracytoplasmic sperm injection (ICSI) are valuable assisted reproductive tools available to clinicians and researchers; however, they primarily focus on producing offspring from subfertile animals (Carnevale, 2008). Stallions (*Equus caballus*) are especially prone to fertility problems, as evidenced by reports that approximately 38% of all prospective stallions fail a breeding soundness examination (Blanchard and Johnson, 1997). The ability to accurately identify aberrant spermatogenic mechanisms that cause reproductive inadequacies may increase the number and efficacy of current treatments available to clinicians and researchers. Therefore, a series of experiments were conducted using the stallion as a model to provide a better understanding of the specific gene products involved in spermatogenesis and useful methods to investigate their functions. Ultimately, this research could lead to the development of new strategies to treat and/or prevent conditions that cause testicular dysfunction.

This dissertation follows the style of *Journal of Andrology*.

Objectives

This research was designed to identify genes associated with differences in spermatogenic capability and semen quality in the stallion testes. Experiment 1 consisted of two studies that evaluated genes that were up-/down-regulated in the testicular parenchyma of three peripubertal stallions of approximately 1.5 yr of age (Study 1A) and two mature stallions >5 yr of age (Study 1B) by microarray analysis. Study 1A employed cDNA microarray technology to investigate gene expression differences in light (spermatogenically active) and dark (spermatogenically inactive) testis parenchyma. Study 1B also utilized cDNA microarray technology to analyze gene expression differences in testicular tissue of two mature horses categorized by either high or low semen quality. The hypothesis of Experiment 1 stated that key genes involved in spermatogenesis and/or steroidogenesis are differentially expressed in testicular tissues from horses of varying spermatogenic states as a result of: 1) regulation by hormones and receptors and/or 2) regulation by paracrine growth factors.

Experiment 2 investigated the localization and concentrations of mRNAs encoding phosphodiesterase (PDE3B) and steroidogenic acute regulatory protein (StAR) genes. The objective of Experiment 2A was to quantify the level of PDE3B and StAR protein mRNA expression in the testis of mature stallions and identify possible correlations between hybridization signal strength (mean silver grain densities) and fertility parameters such as daily sperm production (DSP), apoptosis rate, daily sperm output (DSO), and circulating hormone levels. We tested the hypothesis that PDE3B and StAR protein mRNA hybridization signals are positively correlated with DSP, DSO,

and serum testosterone levels in the mature stallions. Experiment 2B evaluated PDE3B and StAR protein mRNA expression levels in retained and descended testes of six unilateral cryptorchid stallions (three 1-year-olds and three 3-year-olds). In these immature and mature cryptorchid horses, we hypothesized that mRNA levels for PDE3B and StAR protein would differ between the retained and descended testis.

The establishment of an *in vitro* system using whole organ testis slices to study testis function and gene regulation was the emphasis of Experiment 3. The objective was to determine if the precision-cut tissue slice *in vitro* culture system was a viable model to investigate testicular function. The specific aim was to validate the culture systems use as a tool for future investigators to evaluate not only endocrine function, but also gene expression and regulation *in vitro*. In addition, tissue integrity was evaluated microscopically. Viability of cells was evaluated by measuring lactate dehydrogenase (LDH) leakage into medium following culture experiments. Using the precision-cut tissue slice *in vitro* culture system, we evaluated the production and release of steroid hormones testosterone and estradiol 17-β (E_2) and the expression of PDE3B, StAR protein and outer dense fiber of sperm tails 2 (ODF2) genes by the tissues in culture in response to stimulation with varying doses of ovine luteinizing hormone (oLH). We tested the hypotheses that the precision-cut tissue slice *in vitro* culture system may be used to demonstrate a dose response in testosterone levels and up-regulation of a selected LH-induced gene (StAR protein) following treatments with oLH. Collectively, these experiments contribute new information about spermatogenesis and suggest a new technique to study testes function in the stallion.

CHAPTER II

REVIEW OF THE LITERATURE

Stallion Fertility

Stallion infertility can appropriately be defined as the failure to achieve fertilization in a mare. This condition can range from temporary subfertility to individuals that are permanently sterile. Identifying the precise cellular mechanisms involved in stallions with reduced reproductive potential that fall within this range is challenging. Causes are numerous and can be as simple as poor management practices or as obscure as testicular degeneration. In the equine industry, the most reliable means of predicting stallion fertility is the natural breeding of a stallion to large numbers of mares and observing the number of offspring born per mating. However, this practice is not routinely performed due to animal costs and time constraints. It would benefit the industry if a more rapid analysis and efficient method could predict a stallion's reproductive potential.

The purpose of this research is to identify testicular genes in the stallion associated with normal gamete and/or hormone production. Clinically, the future goal is to identify the cause of reproductive failure and treat the animal efficiently so that the full reproductive potential is maximized. Therefore, two important obstacles must be overcome; 1) identification of the etiology responsible for the reproductive disorder and 2) timely corrective measures to increase fertility in a subfertile animal if possible.

In humans, over 60% of male reproductive disorder cases are not identified even with the most comprehensive tests and evaluations available (history and physical

examination, hormone profiles, semen analyses, immunological assays, genetic screening and studies utilizing ultrasound or Doppler) (Patrizio and Broomfield, 1999). It is well known that infertility can affect both males and females alike. However, the results of male infertility in the horse industry are magnified when one stallion has the potential to fertilize up to 100 mares in a breeding season by natural service. According to the most recent release in 2005 from the American horse council, horses have an annual economic impact on the United States of approximately $39 billion with animal numbers reaching over nine million. Although these numbers represent horses of all breeds and ages, many are used for breeding. Reports indicate that stallions are responsible for approximately 65% of low conception rates (Roser, 2001). Additionally, Eisenhauer and Roser (1995) imply a trend towards an increase in subfertility in stallions at breeding facilities. Exact clinical diagnosis of animals with reduced fertility is difficult. Limited therapeutic tools compound the problem often leading to ineffective treatments (Douglas and Umphenour, 1992; Ball, 2008). Identifying the underlying causes of infertility with advances in technology would benefit the welfare of the horse industry. Although interest in stallion fertility has increased over the past years, more research in the area of molecular diagnostics is necessary to enhance the current therapeutic strategies, such as intracytoplasmic sperm injection (ICSI), for infertile and subfertile horses (Carnevale, 2008).

Stallion Reproductive Anatomy and Function

The reproductive system of the stallion encompasses the testes, epididymis, ductus deferens, accessory sex glands and penis. Collectively, these structures produce

and transport spermatozoa and seminal fluid (the ejaculate) to the female. The anatomical development and maturation of these tissues are essential functions and attainment of reproductive capability is dependent upon each.

Testes

The testes synthesize and secrete hormones as well as produce the male gametes. The testis of the stallion is covered by two connective tissue layers, the tunica albuginea and the visceral vaginal tunic, collectively termed the testicular capsule (Senger, 2003). Histologically, a cross section through a testis reveals the parenchyma which is divided into an interstitial compartment and a tubular compartment. The major components of the interstitial compartment are Leydig cells, which produce testosterone, peri-tubular cells, and the vascular supply. The tubular compartment is made up of the seminiferous tubules. Located within the seminiferous tubules are the developing germ cells (spermatogonia, spermatocytes, spermatids) and the somatic Sertoli and myoid cells (Johnson et al, 1997).

Spermatozoa are continually produced in post-pubertal animals with normal testicular development. However, testicular size and activity peaks during the natural breeding season as opposed to the non-breeding season (summer, natural breeding season; winter, nonbreeding season). Variations in testicular cell populations and weight have been well documented in stallions due to age and season (Johnson and Neaves, 1981; Johnson and Thompson, 1983; Johnson and Thompson, 1986). As stallions age, the testicular weight, number of Leydig cells and Leydig cell population per ml of testis volume all increase. In addition, day length affects the daily sperm production (DSP) of

stallions (Johnson et al, 2000b). Seasonal differences in stallions have shown that paired testicular weights in grams, total number of Leydig cells per testis and Sertoli cell numbers were higher in the breeding season compared to the nonbreeding season (Johnson and Thompson, 1986).

Epididymis and ductus deferens

The epididymis is a highly convoluted duct attached to each testis and functions as a spermatozoa storage and maturation organ. Three anatomical regions are recognized in the epididymis; the caput (head), the corpus (body) and the cauda (tail). Spermatozoa and rete fluid exiting the testis enter the caput epididymis via the efferent ducts. In the stallion spermatozoa are retained in the caput for approximately one day where rete fluid is reabsorbed (Senger, 2003). Motility and fertility of spermatozoa begin to be evident as spermatozoa move into the corpus and reside for approximately two days eventually reaching full motility and fertility in the cauda. Stallion spermatozoa are stored in the cauda of the epididymis and can maintain the ability to fertilize an oocyte for several weeks. Spermatozoa stored in the cauda epididymis are transported by smooth muscle contractions of the ductus deferens to the ampulla which leads to the pelvic urethra (Senger, 2003). The urethra is defined as the tube beginning at the bladder and terminates at the glans penis. Both urine and semen (spermatozoa with contributions from the accessory sex glands) exit the stallion through the urethra.

Accessory sex glands

Secretions from the accessory glands (as well as some from the epididymis) provide fluid for spermatozoal transport and constitute the seminal plasma.

Additionally, these secretions provide nutrition and buffers to alleviate hostile acidic conditions found in the female reproductive tract. These contributing glands in the stallion include the vesicular glands, prostate and bulbourethral glands. Limited information is known about the complete function of each accessory gland in the stallion and is beyond the scope of this research.

Penis

The penis is the copulatory organ. The anatomy of the stallion penis is characterized as vascular. Upon sexual stimulation the spongy tissue is filled with blood increasing both the length and diameter of the penis. On the distal end of the penis is the bell-shaped glans. The glans region is highly innervated and is responsible for inducing the ejaculatory process (Senger, 2003). During natural breeding the glans of the stallion penis engorges with blood (a condition called belling) and is anatomically positioned during copulation to be adjacent to the mare's cervical canal so that semen is deposited directly into the uterus.

Endocrinology of Male Reproduction

Hormones synthesized and secreted by the hypothalamus, anterior pituitary and testes, known as the hypothalamic-pituitary-testicular axis (HPT), control male reproductive functions (Roser, 2008). These hormones establish and support germ cell production and regulate the sexual characteristics of males (Matsumoto, 1989). Although steroidal androgens are considered the dominant male hormones, reproductive hormones are not limited to these plasma membrane diffusible molecules. Protein hormones such as gonadotropin releasing hormone (GnRH), luteinizing hormone (LH),

follicle stimulating hormone (FSH), and inhibin bind to receptors located on plasma membranes and are actively involved in male reproduction. Modulation of the HPT on the release of GnRH and the gonadotropins is controlled by feedback loops from hormones such as testosterone, estrogen, and inhibin, which are produced by the testes.

The hypothalamus is responsible for the synthesis and secretion of the decapeptide, GnRH. In the stallion, GnRH is secreted in a pulsatile manner and it regulates the episodic release of LH and FSH from the anterior pituitary (Irvine and Alexander, 1987). Protein hormones are unable to freely diffuse through the external phospholipid bilayer of cells and; therefore, act through second messenger systems. Luteinizing hormone modulates the secretion of testosterone. In the human, it has been established that FSH binds to receptors located on Sertoli cells and causes the release of protein hormones such as inhibin, activin and androgen binding protein (ABP) (Matsumoto, 1989). Similar actions of FSH binding to receptors on Sertoli cells are thought to occur in stallions; however, this has not been proven (Roser, 2001).

Testosterone

Androgens, such as testosterone, are steroid derivatives of cholesterol metabolism. Testosterone is a 19 carbon steroid molecule produced by the interstitial cells of Leydig (Senger, 2003). Testosterone has numerous functions and is essential for maintaining and restoring spermatogenesis in adults, affecting sexual drive (libido), stimulating metabolism, initiating and sustaining male secondary sex characteristics, and supporting organs of the male reproductive tract (Roser, 2001; Martini, 2006). Once secreted, testosterone can either be converted to dihydrotestosterone (DHT), be

aromatized to estradiol, it can suppress GnRH release from the hypothalamus by negative feedback, and it can influence growth of tissues (Senger, 2003). Circulating concentrations of androgens in stallions vary due to season and age. Most reports indicate that mature, fertile stallions (> four-years-old) have circulating testosterone levels that are approximately 0.5 ng/ml in the nonbreeding season and can rise to approximately 2.0 ng/ml during the breeding season (Johnson and Thompson, 1983; Roser and Hughes, 1992a; Roser and Hughes, 1992b, Inoue et al, 1993; Clemmons et al, 1995; Nagata et al, 1998). In addition, it has been shown that DHT follows the same pattern as testosterone, but at lower concentrations, where the ratio of testosterone: dihydrotestosterone was reported at a 6:1 (Bono et al, 1982).

Steinberger (1971) demonstrated that long term administration of low levels of testosterone systemically inhibits the completion of spermatogenesis. Incomplete spermatogenesis due to severely low levels of testosterone in mice was also reported where histological analysis of testis showed meiotic cells and round spermatids, but not elongated spermatids (Kumar, 2005). Studies have also indicated that the direction of action and target of testosterone can augment spermatogenesis in normal mice but suppresses spermatogenesis in juvenile spermatogonial depleted (*jsd*) mice (Meistrich and Shetty, 2003). This negative effect of androgens on FSH and LH secretion reportedly occurs at the level of the hypothalamus (Tilbrook, 1991; Muyan et al, 1993).

Follicle stimulating hormone

The glycoprotein, FSH, stimulates spermatogenesis by binding to receptors located on Sertoli cells. Therapeutically, FSH has been used in the treatment of

infertility, especially in cases where sperm dysfunction has been diagnosed and *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) has been used (Rose et al, 2000). Stimulation of spermatogenesis with FSH has also been used in individuals with azoospermia due to hypogonadotrophic hypogonadism or pituitary failure.

In the stallion, FSH appears more critical during puberty and may not be as significant in adults as previously thought. Circulating levels of FSH in post-pubertal stallions are approximately 6 ng/ml in the breeding season and decline to approximately 4 ng/ml in the non-breeding season (Roser and Hughes, 1992b; Nagata et al, 1998). Roser (2001) reported that *in vitro* culture of testicular tissue from stallions five years of age and older treated with equine FSH showed no effect on steroidogenesis (production and release of T and E_2); however, when treated with 50 ng/ml of equine LH, an increased production of these hormones was observed. In transgenic mice, FSH is not essential for male fertility. However, spermatogenesis is hampered and results in abnormally low sperm output (Kumar et al., 1997). One documented difference between the synergistic roles of testosterone and FSH reported by Zirkin et al. (1994) is that testosterone affects sperm production qualitatively and FSH influences quantity.

Luteinizing hormone

Luteinizing hormone is essential for male fertility. It functions in binding to LH receptors on Leydig cells and subsequently causes testosterone release. Knockout male mice for the LH ligand are infertile and show a significant decrease in testes size, accessory gland size, intra-testicular testosterone level and Leydig cell number (Kumar, 2005). Interestingly, FSH levels are not affected in LH knockout mice compared to

normal controls. Quantification of basal levels of LH in stallions documented in the literature is highly variable due to differences in timing and techniques used to obtain and measure samples. Johnson and Thompson (1983) demonstrate that as age increases in the stallion, circulating concentrations of LH and FSH increase. They also report that these hormones are elevated in the breeding season compared to the nonbreeding season. Luteinizing hormone levels increase on the order of three times more in the breeding season (approximately 6 ng/ml) compared to the non-breeding season (approximately 2 ng/ml) (Roser and Hughes, 1992a; Nagata et al, 1998). Eisenhauer and Roser (1995) reported that stallion testicular tissue treated with 5 ng/ml of equine LH increased testosterone levels in a stallion *in vitro* system 30 fold over basal levels.

Estrogen

The presence of estrogenic compounds in stallions has been known for a long time (Zondek, 1934). However, a function was not discovered until the 1990's when the estrogen receptor knockout (αERKO) mouse was generated (Lubahn et al, 1993). The αERKO model demonstrated that the estrogen receptor and; therefore, estrogen is necessary for male fertility. Hess (2003) reviews how estrogens are metabolized from androgens through the P450 aromatase enzyme in a process termed aromatization.

In the stallion large amounts of estrogens are produced by the testes which make them unique to other males (Raeside, 1969; Eisenhauer and Roser, 1995). The major estrogenic compound produced in the stallion is estrone sulfate (E_1), a conjugated steroid (Raeside, 1969). Circulating levels of E_1 in the stallion fluctuate throughout the season and can range from approximately 100-200 pg/ml (Thompson et al, 1978; Bono et al,

1982; Roser and Hughes, 1992a). Additionally, estradiol 17-β (E_2) is found in high concentration and circulating levels in the stallion have been reported at approximately 150 pg/ml and 50 pg/ml for the breeding and non-breeding season, respectively (Thompson et al, 1978; Nagata et al, 1998). An important function of E_2 is regulation of fluid dynamics in the male tract (Hess, 2003). Estradiol 17-β in the stallion has also been shown to have an agonistic affect on the release of GnRH-induced LH secretion from the pituitary gland (Muyan et al, 1993). Its affect on FSH is thought to be antagonistic since treatments with estradiol or testosterone decreased circulating levels (Thompson et al, 1979).

Spermatogenesis

Spermatogenesis has been defined as the collection of events in the testes that relies on endocrine, paracrine and autocrine regulatory mechanisms to produce spermatozoa (Weinbauer and Wessels, 1999; Johnson et al, 2000). A review of spermatogenesis in the stallion by Johnson et al, (1997) describes the cell types, stages and the changes that take place in the seminiferous tubules which enables the production of spermatozoa from immature germ cells. Briefly, spermatogenesis is categorized into three phases; spermatocytogenesis, meiosis and spermiogenesis. During spermatocytogenesis, the most immature germ cells (spermatogonia) develop. They are populated on the basement membrane of the seminiferous tubules. There are five subtypes of spermatogonia known as A1, A2, A3, B1, and B2 in the stallion (Johnson et al, 1997). These cells grow, differentiate and divide by mitosis until ultimately the B1 spermatogonia divide producing preleptotene primary spermatocytes. Spermatocytes are

capable of undergoing meiosis; therefore, the meiotic phase includes the genetic recombination activity (crossing over) and concludes with the production of haploid spermatids. After the second meiotic division, these cells develop into haploid round spermatids. The final phase is the non-dividing period of spermiogenesis, where round spermatids elongate into spermatozoa. Heninger et al, (2004) describes these cells based on the eight stages of the equine seminiferous epithelium. This developmental process provides a potential for interruption in the production of viable spermatozoa, and subsequently can cause subfertility or infertility. Intense investigation in the area of spermatogenesis has led to an appreciable understanding of its complex components such as proliferation, meiosis, and differentiation. However, the genetic factors critical for the continuous process of spermatogenesis have remained elusive.

Models used to investigate spermatogenic events have included humans, rodents, pigs, and stallions (Peterson and Russell, 1985; Jones and Berndtson, 1986; Wolgemuth et al, 1995). Utilization of the stallion provides a useful model to investigate spermatogenesis. Specifically, the unique spatial initiation of spermatogenesis at puberty, opportunity for frequent testis sample collection by the common management practice of routine castration, and testis size make the stallion an excellent model system.

Initiation of Spermatogenesis in the Stallion

In most mammals, initiation of spermatogenesis occurs at random sites throughout the testis (Clemmons et al, 1995). However, in the stallion, this process begins at the onset of puberty in the central region of the testis and progressively migrates peripherally (Clemmons et al, 1995). Testicular parenchyma of pre-pubertal

animals is dark, reddish-brown in color due to small, closed seminiferous tubules. These seminiferous tubules are incapable of producing mature germ cells and are spermatogenically inactive (Clemmons et al, 1995). At approximately 1.5 yr of age, the stallion reaches puberty and spermatogenesis begins in the central region of the testis proximal to the central vein. Spermatogenically inactive equine testicular tissue can be differentiated from spermatogenically active tissue by visual observation of a gross change in color from dark, reddish-brown to a light parenchymal color. The observed shift in tissue structure and color is due to the formation of larger tubules as well as spatial changes in Leydig cells, which ultimately results in spermatogenic activity (Clemmons et al, 1995). This system provides an excellent model to study spermatogenesis wherein, both spermatogenically inactive (dark) and active (light) tissue can be evaluated in the same animal under the same environmental conditions and same hormonal milieu. Currently, there are no other known livestock species that exhibit similar physiological changes within the testis.

Cryptorchidism

Embryonic testicular development occurs in the abdomen. However, for normal spermatogenesis to proceed in mammals, a hypothermic environment compared to normal body temperature is essential for gamete production. Testicular cooling is initiated by descent into the scrotum where scrotal temperature is lower than the temperature in the body cavity. In the horse, core body temperature is approximately 38°C and scrotal temperature is approximately 32°C (Green et al, 2005; Staempfli et al, 2006). In the colt, descent through the inguinal ring occurs between 300 days of

gestation and 10 days after birth (Bergin et al, 1970). Occasionally, one or both testes fail to completely descend into the scrotum. This condition is termed unilateral or bilateral cryptorchidism, respectively, and occurs in several species. However, it is most frequent in the equine and porcine species (Bergin et al, 1970).

In humans, cryptorchidism is considered to be the most frequent reproductive disturbance in newborns that requires surgical and medical assistance (Bilinska et al, 2003). This condition has been used as a model to study spermatogenic and steroidogenic functions in several species such as mice (Meistrich et al, 1973), rats (Shakeel et al, 2001), bulls (Volger et al, 1991), rams (Mieusset et al, 1991), boars (Raeside et al, 1988) and stallions (Blanchard et al, 2000). In stallions and humans, previous studies have demonstrated that cryptorchidism is associated with reduced fertility through elevated testicular temperatures incompatible with spermatogenesis (Cox and Williams, 1975; Stickle and Fessler, 1978; Hayes, 1986; Hejmej and Bilinska, 2008). Arighi et al, (1987) compared stallions with normal scrotal descent of testes to those with inguinal and abdominally retained testes and showed that mean weight, size, layers of germs cells present and tubular diameter were all increased in scrotal testes. Histological evidence revealed full spermatogenic development in the scrotal testes; however, development was arrested in early spermatocytes in tissue retained in or at the inguinal canal. Testes retained in the abdominal cavity had no germ cell development past spermatogonia. One report states that elevated abdominal temperature does not decrease hormone synthesis since Leydig cell numbers are increased in abdominal compared to scrotal testes (Araghi et al, 1987). This increase, not only in interstitial

cells but in the amount of interstitial space as well, resulted in higher circulating concentrations of estrone sulfate and testosterone in these stallions. Others report that when stallion testicular temperatures were experimentally elevated by insulating the testes, semen quality, sperm motility, concentration and number of ejaculates decreased (Blanchard et al, 2000). In the same study, circulating testosterone levels decreased and LH levels increased after removal of thermal insult. Although the morphological changes in cryptorchids are well established, the cellular and molecular changes causing testicular degeneration is not fully understood.

Methodologies for Evaluating Fertility in Stallions

The limited number of tests available to clinically assess fertility in stallions has stimulated a demand for researchers to investigate additional methodologies. One of the roles of research is to create and/or validate procedures that can be added to the current repertoire of tests for clinicians. Clinically, the most desirable and definitive test for evaluating a stallion's fertility is through breeding trials where an individual stallion is bred to a cohort of mares and conception rate and/or live birth percentages are determined. However, this may not be economically feasible to clientele.

The most commonly evaluated diagnostic methods used clinically are physical examinations of the reproductive system and ejaculate assessments for sperm motility, sperm number, and sperm morphology because these are non-invasive procedures and are quick (Ball 2008). In addition, some veterinarians are incorporating multiple tests along with the previously mentioned common assessments such as ultrasonography and the utilization of endoscopy of the urethra to evaluate lesions and/or infections of the

accessory sex glands (Ball 2008; Varner 2008). General methods used to evaluate stallion semen include total motility which is an estimation of the percentage of spermatozoa that demonstrate movement and progressively motile spermatozoa (PMS) which indicate only those cells that are actively moving forward or in a path with circular motion (Ball 2008; Varner et al, 2008). Other general methods used to evaluate fertility are sperm concentration, DSO, sperm morphology, and the sperm chromatin structure assay (SCSA) (Johnson et al, 2000; Thompson et al, 2004; Love 2005). Sperm concentration is most often determined by counting cells with the aid of a hemocytometer, or by photometric or fluorescence analysis (Varner et al, 2008). DSO can be measured as actual or predicted daily sperm output. The actual DSO is determined by multiple daily semen collections (minimum of seven consecutive days) and the reported value is the mean of the final three days of semen collections. Predicted DSO is determined by measuring testicular dimensions by ultrasound and calculating values using prediction equations (Varner et al, 2008). It has been estimated that in sexually mature stallions the DSO is around five billion spermatozoa per day; however, season and age can cause this number to fluctuate (Thompson et al, 2004). Hormone profiles for T, E_2, FSH, LH and inhibin are assessed clinically and are critical in understanding fertility. Although reports in the literature of basal hormone concentrations in stallions are inconsistent, the variation is mostly attributed to radioimmunoassay methods across different laboratories (Thompson et al, 1979; Irvine and Alexander, 1987; Stewart and Roser, 1998). In addition, these hormone levels vary among horses of different ages and samples collected during different seasons. A recent

tool that has become available to clinical practitioners is the sperm chromatin structure assay (SCSA). SCSA enables the evaluator to assess DNA damage in sperm cells based on the amount of dye incorporated into single stranded DNA (ssDNA) or double stranded (dsDNA). Reports in some species have indicated that sperm cells with high concentrations of fragmented DNA (ssDNA as opposed to dsDNA) are related to subfertility (Love, 2005). The limitation to frequent use of SCSA is the need for a flow cytometer in the laboratory.

Techniques utilized in research for the purpose of evaluating infertility include the assessment of DSP and apoptotic rate. These tests are more invasive, can require whole testis samples, and may cause irreversible damage to the testicular tissue. Additionally, these procedures are often labor intensive and the results from tests may not be available for several days. However, research methodologies utilized to investigate infertility are valuable tools as they provide insight into the mechanisms that regulate testicular function. Daily sperm production is a calculation of the number of spermatozoa produced by both gonads in a day and is a good predictor of testicular function. However, DSP is an invasive technique commonly assessed by estimating the number of mature spermatid nuclei seen by phase-contrast cytometry after homogenization of testicular tissue (Amann et al, 1976; Johnson and Neaves, 1981). Johnson and colleagues (2000) characterized daily sperm production per gram of parenchyma at different developing stages of spermatogenesis for several species. The rate of germ cell death and specific hormone levels can also be used as a diagnostic tool for understanding fertility. Apoptosis, or programmed cell death, is the intentional

removal of cells from specific tissues. This ATP requiring mechanism has been well documented in the testis and in several species including the stallion (Santos et al, 1999; Lue et al, 2002; Heninger et al, 2004). In cases where testicular degeneration is a concern, research options like apoptosis studies, tissue biopsies, microarrays, *in situ* hybridization studies, and tissue culture can evaluate aberrant functions in the testis. One example is the work by Heninger and colleagues (2004) that demonstrated significantly higher rates of apoptosis in stage IV and V stallion seminiferous tubules. However, the procedures used to determine these valuable results required whole testis samples of normal stallions which would not be used in clinical diagnosis. It does; however, contribute greatly to our knowledge of how spermatogenesis occurs.

Microarrays

Advancements in biotechnology have provided numerous methods for the detection and quantification of gene expression. Until recently, gene expression studies evaluated differences between two or more cells at varying time intervals, treatment conditions or in different tissues using traditional methods such as northern blots, differential display-reverse transcription polymerase chain reaction and sequencing of cDNA libraries (Kamberova and Shah, 2002). However, the vast amount of information provided by complete sequencing of genomes has demanded a better solution to evaluating larger numbers of gene products more efficiently. One tool that enables researchers to investigate a large number of gene products that are up- and down-regulated within a system is the microarray. It has been rapidly utilized in the fields of biological and medical sciences as well as in the pharmaceutical industry (Rockett et al,

2001a). Other names include DNA arrays, complementary DNA (cDNA) arrays, and genome chips (Rockett and Dix, 1999). Prior to the introduction of microarray technology the conventional experiments available were time consuming and severely limited the number of genes to be investigated in a single experiment. Microarray technology overcame this obstacle which allowed for high through-put screening of thousands of gene products simultaneously in one experiment (Schena et al, 1995).

Microarrays are ordered spots of DNA probes attached to solid surfaces such as glass slides or nylon (Kamberova and Shah, 2002). The DNA molecules can either be oligonucleotides (short sequences ranging from 20 to 60 base pairs in length) or cDNAs, which are typically much longer and allow for more specificity. Oligonucleotides, or cDNAs, correspond to a specific gene sequence and are spotted and attached to the surface of slides. A microarray experiment first begins by isolating mRNA from test samples and labeling each with a different fluorescent dye. The samples are allowed to hybridize in parallel to DNA sequences immobilized onto a solid surface (Schulze and Downward, 2001). Transcripts can then be simultaneously detected and quantified by fluorescent scanning instrumentation by measuring the amounts of specific fluorescence at each coordinate.

Microarray technology has become a popular tool in analyzing spermatogenic function and regulation based on the number of investigators using array methodology (Rockett et al, 2001b; Tanaka et al, 2002; Sha et al, 2002; Ohta and Fuse, 2002; Schultz et al, 2003; Pang et al, 2003; Yu et al, 2003; Almstrup et al, 2004; Hoffer et al, 2005). Employment of arrays has opened the field to greater knowledge of multiple gene

interactions and timing of events during the developmental processes of maturing male germ cells. Canadian researchers isolated three different spermatogenic cells (spermatocytes, round spermatids and elongated spermatids) of adult Sprague-Dawley rats and found that of 216 gene probes on the commercial array used, 96 were expressed in those cells (Aguilar-Mahecha et al, 2001). Additionally, only 50% of the expressed genes were found in all three cell types evaluated and only 25% were detected in both spermatocytes and round spermatids. This leads to the observation that there must be specific genes under transcriptional and translational control that dictate germ cell maturation in the testis. Others have used microarrays to identify specific cells in the testes that are hormonally regulated (McLean et al, 2002) or to determine which genes are over expressed or under expressed in two different testicular germ cell tumors (Hoffer et al, 2005).

Commercial microarrays have been available for multiple species such as humans and rodents since 2000 (Rockett et al, 2001a; Sha et al, 2002). Recently, equine specific microarrays have been added to this list which are available to the public (see reviews, Chowdhary and Raudsepp, 2008; Ramery et al, 2009). Prior to the availability of equine specific microarrays, in order to use microarray technology to investigate gene expression in equine tissues, cross-species hybridization using long cDNA microarrays was utilized (Ing et al, 2004). Cross-species hybridization has proven valid in cDNA microarray experiments where porcine RNA was hybridized to human nylon microarrays (Moody et al, 2002). Using this methodology, we hybridized both dark and light testicular parenchyma of peri-pubertal stallions and tissue from two mature

stallions that differed in semen quality parameters to human cDNA targets (Ing et al, 2004). These experiments identified important transcripts that were up- or down-regulated in the different tissue and narrowed the focus on key regulators involved in spermatogenic function.

Genes of Interest

Although many genes have been implicated in pathways resulting in normal and abnormal testicular function, this project involved evaluating the gene expression of three transcripts in the male testes; PDE3B, StAR protein and ODF2. All three were found to be differentially expressed on at least one of the microarrays conducted in these experiments. In addition, StAR protein and ODF2 mRNA have previously been identified in other reported testes specific microarray results thereby validating their importance in male reproduction (Aguilar-Mahecha et al, 2001; Sha et al, 2002; McLean et al, 2002). To our knowledge, PDE3B specifically has not been previously reported in other testes specific microarrays, although several phosphodiesterase family members have.

Phosphodiesterase

An important pathway responsible for eliciting a cellular response involves signal transduction by intracellular second messengers such as cyclic 3', 5'-monophosphate (cAMP) and cyclic 3', 5'- guanosine monophosphate (cGMP) (Conti, 2000). Theses second messengers are tightly regulated through the synthesis of adenylyl and guanylyl cyclases, via activation through hormone interactions with membrane bound G protein coupled receptors, and by the rate of hydrolysis through PDEs (Fawcett

et al, 2000). Cyclic nucleotide phosphodiesterases are enzymes that hydrolyze cyclic nucleotides, thereby, terminating their downstream activities and have been implicated in negative feedback mechanisms that control these second messengers (Park et al, 2002).

The PDE enzymes are structurally related; however, they differ in sequence, chemical properties, inhibitor responses, regulatory mechanisms, and cellular localization (Miki et al, 1996). Currently, eleven families (PDE1-11) have been identified and are classified into five groups according to their substrate affinities, which include cAMP-specific, cGMP-specific, Ca2+/calmodulin-stimulated, cGMP stimulated, and cGMP inhibited PDEs (Welch et al, 1992). All PDEs to date have a conserved ~270 amino acid sequence in the catalytic domain of the enzyme and differ in their N-terminal domains (Beavo, 1995).

One particular family, the type III PDE isoenzymes also known as cyclic GMP-inhibited PDEs, is unique in that those PDEs hydrolyze both cGMP and cAMP. However, the hydrolysis of cAMP is inhibited by low concentrations of cGMP, speculated to be caused by competitive binding. Specific to this family is a 44 amino acid insert in their conserved domains, which does not align with other PDEs, and are designated as PDE3A and PDE3B (Miki et al, 1996). The two members of the PDE3 family are unique in their binding ability for both cAMP and cGMP; however, the V_{max} for cGMP is lower than that of cAMP (Manganiello et al, 1995). Although PDE3A and PDE3B are similar, they are distinct genes that map to different chromosomes. PDE3A maps to human chromosome 12 and equine chromosome 6; whereas, PDE3B maps to

human chromosome 11 and equine chromosome 7 (Miki et al, 1996). Gene expression studies of PDE3A and PDE3B have shown that these two isoforms are found in different tissues in the body. PDE3A has been found in the cardiovascular system, smooth muscle cells of the gastrointestinal tract, and oocytes. PDE3B is highly expressed in adipose tissues, hepatocytes, renal epithelium, and in the cells of the seminiferous tubules (Reinhardt et al, 1995).

The members of the PDE3 family are activated through phosphorylation by products that increase intracellular cAMP concentrations, such as forskolin, and are specifically sensitive to inhibitors such as milrinone and cilostamide (Miki et al, 1996; Conti, 2000). The majority of our understanding of PDE3B has been through its link to the insulin pathway, whereby, phosphorylation of the enzyme regulates the effects of insulin on lipid metabolism. One theory suggests that low levels of PDE3B in patients with diabetes cause an increase in lipolysis and acidosis (Beavo, 1995; Zhao et al, 1997; Harndahl et al, 2002).

Butcher and Sutherland (1962) were the first to describe cAMP. Included in their discussion was the discovery of specific enzymes reported to degrade cAMP. These enzymes were later identified as phosphodiesterases. In this discussion, it was suggested that hormones might control the rate of cyclic nucleotide degradation. Based on these findings, investigators began to examine the link between PDEs and their role in the reproductive system. One of the preliminary studies of PDE involvement in reproduction was conducted in *Drosophila melanogaster*. In this study, investigators reported that in flies, mutations encoding the PDE gene at the *dunce* locus resulted in

female sterility (Saltz and Kiger, 1982). Later studies demonstrated that FSH in rats caused not only an increase in adenylyl cyclase leading to an increase in cAMP, but also stimulated an increase in production of PDE activity as well (Conti et al, 1983). More recently, knockout mice for PDE3A, PDE3B and PDE4D have been generated. Interestingly, PDE3A knockout female mice are sterile due to the lack of cAMP hydrolysis. In this study, oocytes were arrested in prophase I of the first meiotic division and could not resume maturation due to the elevated cAMP levels (Wiersma et al, 1998; Masciarelli et al, 2004). PDE4D null mice also display an obvious phenotype that results in smaller bodied mice and females exhibit a 75% reduction in ovulation rate and reduced fertility (Jin et al, 1999). Interestingly, PDE3B knockout mice are fertile but demonstrate reduced gonadal adipose tissue and are overall leaner than wild-type mice indicating that PDE3B may be involved in energy metabolism (Choi et al, 2006). To date, the overall effects of PDE3B on spermatogenesis have not been investigated in PDE3B knockouts. These studies indicate the importance of PDEs in mice gametogenesis.

Although most of the progress on PDE function in reproduction has been learned through studies in the female, important implications have been realized in the male that contribute to the need for further investigation. Dibutyryl-cAMP, a cAMP analog, was first shown to inhibit mitosis *in vivo* at the level of the testis when given to rats (Hollinger and Wang, 1974). This study implied that there is a necessary mechanism for decreasing cAMP levels for appropriate germ cell maturation, and suggested that one of the PDEs was involved in this pathway. Later, Verhoeven and colleagues attempted to

show that FSH decreased PDE activity; however, their hypothesis was proven incorrect when they reported that FSH and isoproterenol stimulated PDE activity in cultured rat Sertoli cells (Verhoeven et al, 1981). At this time, it was realized that several forms of PDE were present in the testis, and there were different concentrations of PDEs that were dependent upon germ cell development. Subsequent studies using immature rats demonstrated that FSH increased PDE levels in the seminiferous tubules but not in the interstitial space (Conti et al, 1983). In order to show that different PDEs act in the testis, Yan and colleagues (2001) evaluated the expression of PDE1A, PDE1B, and PDE1C genes. Results of their investigation showed that PDE1A mRNA was found in round to elongated spermatids, PDE1C mRNA was found in stages with germ cells undergoing meiotic divisions, and PDE1B mRNA was found in both the seminiferous epithelium and the interstitial space of the testis.

Of the eleven PDE family members, eight have been localized to the testis by *in situ* hybridization and include varying isoforms of the PDE1, PDE2, PDE3, PDE4, PDE7, PDE8, PDE10, and PDE11 families (Morena et al, 1995; Soderling et al, 1999; Soderling and Beavo, 2000; Sasaki et al, 2002). Based on previous research and localization of the PDE3B gene, we expected to find it more abundantly expressed in testis tissue that is actively involved in spermatogenesis. Further investigation of this particular enzyme function in the testis will be beneficial for unmasking its regulatory role in spermatogenesis.

Steroidogenic acute regulatory protein (StAR)

In contrast to PDEs, the role of StAR protein in the testis has been well

characterized. The rate-limiting step in steroid synthesis occurs in the mitochondria where cytochrome P450 cholesterol side-chain cleavage enzyme complex (P450ssc) converts cholesterol to pregnenolone (Miller, 1988; Clark et al, 1994; Stocco and Clark, 1996). P450ssc resides on the inner mitochondrial membrane and requires cholesterol to be transported from the outer to the inner membrane to initiate steroidogenesis (Kerban et al, 1999). Cholesterol delivery to the inner mitochondrial membrane is accomplished by StAR protein. It has been confirmed as the translocating enzyme through knockout mice and cell culture studies (Sugawara et al, 1995; Caron et al, 1997). Arguably, the most important work demonstrating that StAR is crucial for steroidogenesis is the human condition lipoid congenital adrenal hyperplasia. This condition results from a mutation in the StAR gene causing a defect in the cholesterol transporting ability of the enzyme. Individuals with this autosomal recessive, potentially lethal disorder are unable to produce steroids and often have large accumulations of cholesterol in the body (Reyland et al, 2000). Knockout mice for StAR protein show similar traits to the human condition with substantial lipid deposition in the adrenal gland and a reduction in corticosterone production (Manna and Stocco, 2005).

StAR protein gene transcripts have been localized in tissues that synthesize steroids such as the adrenal glands, ovary, brain, placenta, and testis (Pollack et al, 1997; Manna and Stocco, 2005). The StAR gene is induced by LH through a second messenger pathway. Gene expression studies have demonstrated increased mRNA levels within 30 minutes after stimulation with human chorionic gonadotropin (hCG) with continual increase for 6 hr (Manna et al, 2004). Currently, the gene encoding StAR

protein has been of interest for patients with Alzheimer disease (AD). Investigators report that patients with AD have an increased level of StAR protein and LH receptor expression in specific neuronal regions of the brain compared to controls (Webber et al, 2006). Our studies are expected to demonstrate a decrease in StAR protein gene expression levels in horses with reduced fertility parameters.

Outer dense fiber of sperm tails 2

In mammalian species, sperm motility is controlled by the flagellum and a recent review describes its components and ultrastructure (Turner, 2003). Briefly, the flagellum is comprised of the connecting piece, midpiece, principle piece and end piece. A distinguishing characteristic of the midpiece is the existence of accessory structures termed outer dense fibers (Fawcett et al, 1975). There are nine outer dense fibers (identified as ODF1 – ODF9) which surround the axoneme and are in close proximity to the mitochondria of the sperm tail. Suggested functions of these fibers have been for protection during transport, elasticity of flagellum and general structural support of the flagellum (Peterson et al, 1999). ODF2 has been isolated and has been specifically localized in round spermatids of rats (Brohmann et al, 1997). Beyond the localization and apparent structural function of ODF2 in specific species, relatively little is known about this protein. Interestingly, microarray data support the evidence that it is one of the most highly up-regulated gene targets in the spermatogenically active testicular parenchyma of peri-pubertal stallions and may be a possible predictor of highly fertile animals (Ing et al, 2004).

Testicular Culture

Our knowledge of spermatogenesis and the cellular communications that regulate the process has been greatly enhanced over the past century through the use of *in vitro* culture systems. Multiple reviews clearly state their usefulness in understanding pathways, modification of germ cells and treatment of male infertility (Orth et al, 1998; Parks et al, 2003). Their clear advantage is the establishment of controlled environments which enable researchers to test for specific mechanisms without compromising normal structural relationships. However, their use has proven problematic due to an inability to maintain cell viability for extended periods of time and the need to supply uniform access of media and compounds to the tissues.

Precision-cut tissue slice (PCTS) in vitro culture

The first described tissue culture system for investigating testicular cells utilized rabbit testis in blood plasma (Champy, 1920). Since that time, there have been numerous advances in the use of different types of culture systems for *in vitro* studies of spermatogenesis (Staub, 2001). One of the more promising systems for evaluating spermatogenesis *in vitro* utilizes slivers of whole organs placed on wire mesh grids and incubated in culture vessels (Smith et al, 1985). The advantage of the precision-cut system is the reproducibility from mechanical tissue slicing, the ability to use organ slices that mimic systems *in vivo*, and the maintenance of tissue architecture (Parrish et al, 1995). To date, this culture system, known as the precision-cut tissue slice (PCTS) *in vitro* culture system, has been used successfully in several tissues such as liver, kidney, lung and heart (Parrish et al, 1995; Turney et al, 1999). In addition, the multiple animal

models that have used the PCTS *in vitro* culture system to investigate physiological responses in tissue help strengthen the possible use of this tool as a viable testicular culture system. These studies have included *in vitro* challenges of various compounds on mice liver slices, rabbit renal slices and human prostate slices (Parrish et al, 1999a; Turney et al, 1999; Schmelz et al, 2001; Parrish et al, 2002). This research report intends to demonstrate that this system is a capable tool for investigation of whole testicular stallion tissue. Validation of this *in vitro* system for testicular tissue will facilitate studies to investigate mechanisms which regulate expression of genes that are involved in spermatogenesis. Ultimately this system could be used to answer some of the undefined questions in male fertility and subfertility in all mammalian species.

CHAPTER III

DIFFERENTIALLY EXPRESSED GENES IN TESTICULAR TISSUE OF STALLIONS OF VARYING SPERMATOGENIC CAPABILITY AND SEMEN QUALITY*

Introduction

The initiation of spermatogenesis begins with a gradual series of events that transform the relatively inactive male gonad into an organ that produces high levels of testosterone and mature spermatozoa. Spermatogenesis in the colt (young stallion, an intact male) initiates in the central region of the testis around the second year of life and spreads peripherally until the entire testis is spermatogenically active. Spermatogenic activity can be noted grossly by the light tan color of the central testicular parenchyma in the 1.5-year-old horse, while peripheral tissues of the testis are dark red-brown (Clemmons et al, 1995). Histologically, dark testis tissues are laden with Leydig cells and large macrophages in the interstitial spaces and have small seminiferous tubules that are not producing sperm and that occupy no more than 50% of the parenchyma (Clemmons et al, 1995). In contrast, light testis tissues have reduced densities of Leydig cells, macrophages, and other interstitial cells as the seminiferous tubules (which are composed primarily of nonpigmented cells) increase in size and cell density to compose

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* Reprinted and modified with permission from "Gene Expression in the Spermatogenically Inactive ''Dark''and Maturing ''Light'' Testicular Tissues of the Prepubertal Colt" by NH Ing, AM Laughlin, DD Varner, TH Welsh, JR., DW Forrest, TL Blanchard, L Johnson, 2004. *Journal of Andrology*, 24:535-544, Copyright 2004 by The American Society of Andrology.

Approximately 70% of the testis parenchyma. Thus, the prepubertal colt testis presents an excellent model for functional genomics to identify genes that are turned on or off during the initiation of spermatogenesis. Its adjacent dark and light tissues have similar organizational components, including Leydig cells, seminiferous tubules, and influences of systemic hormones, but they differ in tubule maturity and function; namely, the initiation of spermatogenesis in the light tissue (Clemmons et al, 1995). The long-term goal of this study is to better understand the gene regulation critical to spermatogenesis and to identify new therapeutic approaches to enhance male fertility.

We hypothesize that key genes are involved in spermatogenesis and/or steroidogenesis and are differentially expressed in testicular tissues from horses of varying spermatogenic states as a result of regulation by hormones, growth factors and receptors. Initially, 12 gene products were chosen that were predicted to be differentially expressed in dark and light testis tissues. These gene products included androgen receptor, progesterone receptor, estrogen receptors alpha and beta, steroid hormone receptor coactivator 1, high-density lipoprotein binding protein, transforming growth factor (TGF) beta 1, 2, and 3, oxytocin receptor, 33lyceraldehydes phosphate dehydrogenase, and c-fos. Of the 12, androgen receptor and TGF beta 1 appeared to be differentially expressed, but this was not confirmed on *in situ* hybridization or Northern blots.

For this reason we turned to microarrays on glass slides in order to simultaneously and efficiently compare levels of expression of 9132 gene products between matched dark and light testis tissues of three colts (Study 1A) and testicular

parenchyma of two mature stallions (Study 1B) of which one was classified as fertile and the other subfertile based semen evaluation (Rockett et al, 2001b; Schultz et al, 2003). *In situ* hybridization and Northern blot analysis confirmed and localized the differential regulation of gene products chosen for these analyses. These discoveries open investigations into novel pathways that regulate spermatogenesis. Importantly, these data also demonstrate that analysis of equine gene expression can be performed using human cDNA microarrays because of a high level of sequence conservation. This conservation is evident in analyses of 193 horse cDNAs from a testis library that displayed an average of 92% identity between horse and human sequences (L. Skow, personal communication).

Materials and Methods

Animals and sample preparations

Seven 1.5-year-old colts and two mature stallions were castrated by standard surgical means under appropriate anesthesia. Testes were cut midsagittally, and gross dissection of dark (where appropriate) and light tissues from each testis preceded mincing, snap freezing in liquid nitrogen, and storage at -80°C. Five left testicular samples were used for microarrays, four different testes were used for Northern blot analyses, and all seven colt samples were used for *in situ* analyses. Colt testes samples were consistent across all horses with regard to age and presence of meiosis, as indicated by spermatocytes in light tissues. PolyA+ RNA was isolated directly from dark and light tissue samples from all seven young horses and from the two mature stallions with the Fasttrack kit (Invitrogen, San Diego, CA). Testes were fixed in 4% paraformaldehyde in

phosphate-buffered saline (PBS) (pH 7.2) and embedded in Paraplast (Fisher Scientific, Houston, Tex) for *in situ* hybridization. Reagents were of molecular biology grade and purchased from Sigma Chemical Company (St Louis, MO), unless otherwise noted. All animal procedures were approved by the Texas A&M University Animal Care and Use Committee.

Microarrays

Poly A+ RNA samples from all horses appeared of high quality and purity on an ethidium bromide–stained Northern gel (results not shown). Testis RNA samples (1.8 mg each) were reverse transcribed and labeled with 5'Cy3 and Cy5 fluors, respectively; hybridized to immobilized human cDNAs of the UniGEM Human V 2.0 microarray; and analyzed by Incyte (St Louis, MO). Probe labeling reactions were incubated at 37°C with 200 ng of polyA+ RNA, 200 units M-MLV reverse transcriptase (Life Technologies, Gaitherburg, Md), 4 mM DTT, 1 unit Rnase Inhibitor (Ambion, Austin, TX), 0.5 mM dNTPs, and 2 mg of labeled 9-mers for 2 hours. Sample amplification was not used in order to decrease artifacts in expression ratios. The probe samples were then combined, purified, precipitated, and allowed to competitively hybridize to a single microarray. Briefly, the hybridization protocol consisted of the following: resuspension of probe solution with a five minute incubation at 65°C, application to array, cover slipping, and sealing in an evaporation chamber for 6.5 hours at 60°C. After hybridization, the glass slides were washed in three consecutive washes of decreasing ionic strength. The microarrays consisted of 9132 genes generated from polymerase chain reaction (PCR) products (15% were expressed sequence tags) and included 190

controls. The fluorescence data was converted to ‘‘balanced difference’’ values (balanced to account for differences in fluorescence labeling between the cDNA pools) with GEMTools 2.4 (Incyte Pharmaceuticals Inc, Palo Alto, CA). In Study 1A, balanced differences with positive values are ratios of signals from the dark testis tissue to light testis tissue (Cy3/Cy5). Negative balanced difference values indicate that the ratio is inverted, with the larger light testis tissue (Cy5) signals divided by the lesser dark tissue signals (Cy3). Positive values in Study 1B represent balanced differences of genes more highly expressed in the subfertile stallion and negative values indicate genes more highly expressed in the fertile animal. Normalization was performed per chip to a series of internal controls including yeast cDNAs and fluorescent standards by gridding and region detection algorithms. Gene products with balanced difference values of equal to or more than 1.7 or less than -1.7 were considered differentially expressed in both studies ($P < .05$) (Yue et al, 2001; Moody et al, 2002; Reynolds, 2002). Spots failing to meet minimal criteria (signal strength 2.5 times that of background and covering at least 40% area of the spot) were given balanced difference values of 0 and were excluded from evaluation. Differentially expressed genes were identified with GeneSpring 4.0.2 software (Silicon Genetics, Redwood City, CA).

Cloning of down-regulation of ovarian cancer 1 (DOC1), golgi apparatus protein 1 (GLG1), cell division cycle 2 (CDC2), steroidogenic acute regulatory protein (StAR) cDNAs from horse testis

To make high-specificity probes for androgen receptor (AR), down regulated in ovarian cancer 1 (DOC1), Golgi apparatus protein 1 (GLG1), cell division cycle 2

(CDC2), and steroidogenic acute regulatory protein (StAR) mRNAs of the horse, cDNAs were cloned from horse testis, as previously described (Ing et al, 1996). PolyA+ RNA (200 ng) was reverse transcribed at 42°C with Superscript II (Life Technologies, Newark, NJ) and random hexamer primers. PCR was performed twice with two sets of primers designed from human cDNA sequences with the set of primers used for the second amplification nested inside the first. cDNAs were cloned into the PCR2.1 vector (Invitrogen, Austin, TX), and multiple clones were sequenced. The horse DOC1, GLG1, CDC2, and StAR protein cDNA sequences are in GenBank (accession numbers AY349169, AF547432, AF547431, and AY349170, respectively).

Northern analysis of dysferlin (DYS), DOC1, outer dense fiber of sperm tails (ODF2), phosphodiesterase 3B (PDE3B), and 37lyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNAs

PolyA+ RNA samples (5 mg) were analyzed on a denaturing (Northern) gel, as previously described (Ing et al, 1996). RNA Millennium Markers from Ambion (Austin, TX) were run alongside. The RNA on the gels was transferred to nitrocellulose membranes and the blots were hybridized to radiolabeled antisense cRNA probes produced by *in vitro* transcription (T3 RNA polymerase) with the Maxiscript kit (Ambion, Austin, TX) using [32P]-UTP (3000 Ci/mmol; New England Nuclear, Boston, MA). The cDNA template for DOC1 was a PCR product (described above), while those for dysferlin (DYS), outer dense fiber of sperm tails (ODF2), phosphodiesterase 3B (PDE3B), and GAPDH were constructed from linearized plasmids as follows. Human DYS plasmid (pINCY-4462162 vector; Incyte Genomics, Palo Alto, CA) was linearized

with *Eco*R1 and transcribed with T7 RNA polymerase; rat ODF2 plasmid (pBluescript II vector) (GenBank accession number U62821) was linearized with *Nco*1 and T7 RNA polymerase; human PDE3B plasmid (pBluescript II vector) (GenBank accession number U38178) with *Bam*H1 and T3 RNA polymerase; and ovine GAPDH plasmid restricted with *Bam*H1 and T7 RNA polymerase. After stringent washing, the blots were exposed to x-ray film (XAR, Kodak, Rochester, NY). Densitometry with Intelligent Quantifier software (Bio Image, Ann Arbor, MI) was used to compare hybridization signals between samples and reported as an average fold change in densitometric units across four horses. Individual gene expression units were normalized to GAPDH to account for loading differences.

In situ hybridization

The mRNA for DYS, ODF2, and DOC1 were localized on serial cross sections from the left testes of 1.5-year-old horses by *in situ* hybridization analysis, as described previously (Ing et al, 1997). Tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated using *in vitro* transcription with α[35S]-UTP (1250 Ci/mmol, New England Nuclear, Boston, MA). The human DYS plasmid (pINCY-4462162 vector, Incyte) linearized with *Eco*RI and transcribed with T7 RNA polymerase to produce antisense cRNA. In addition, negative control sense cRNA from DYS was produced from *Not*1 and SP6 RNA polymerase. Rat ODF2 and human PDE3B cDNAs (GenBank accession numbers U62821 and U38178) were restricted within the cDNAs with *Nco*1 for ODF2 and with *Bam*H1 for PDE3B and transcribed with T7 or T3 RNA polymerase for antisense and sense cRNAs. Antisense and sense horse DOC1 cRNAs

were produced from PCR products (described above) with T3 and T7 RNA polymerases, respectively. After hybridization, washing, and ribonuclease A digestion, slides were coated with photographic emulsion (Eastman Kodak, Rochester, NY) and developed two or four weeks later, depending on hybridization signal strength. Cell nuclei were counterstained with 0.1% toluidine blue. Micrographs were captured on a Zeiss Axioplan 2 Microscope (Gottingen, Germany) using Adobe Photoshop (Adobe Systems, Seattle, WA).

Results

Microarray analyses identify a wide variety of expressed genes

The complete data sets (Study A1) from the microarray studies are available online at http://animalscience.tamu.edu/ning/microarraydata/horse1.html for ‘‘horse1’’ and adjacent websites for ‘‘horse2’’ and ‘‘horse3’’ (summarized in Table 1). Genes are grouped and ranked by average balanced difference values. Two clear results generated from the microarray analysis determined that the majority of genes were not differentially expressed between dark and light tissues of the testes, and individual horses had unique differences in their patterns of testicular gene expression. However, there were a number of genes that appeared to be preferentially expressed in dark or light tissue. DYS, DOC1, and GLG1 genes were preferentially expressed in dark tissue, while ODF2 and PDE3B genes were more highly expressed in light testis tissue of all 3 horses analyzed by microarrays (>1.7 balanced difference value, Incyte GEMTools software).

Table 1. Differentially expressed genes in dark/ light testis of prepubertal colts.*

DARK-SPECIFIC

Signaling Molecules

3.6 – Insulin-Like Growth Factor 2 (IGF2)
3.6 – Parathymosin–Cell Cycle Regulated (PTMS)
2.1 – ATP-Binding Cassette 3 (ABCA3)
1.9 – CDC-Like Kinase 1 (CLK1)
1.9 – Major Histocompatibility Complex, Class 1, B (HLA-B)
1.8 – Insulin-Like Growth Factor Binding Protein 7 (IGFBP7)
1.8 – Protein Kinase D1 (PRKD1)
1.8 – CD63 Molecule (CD63)
1.7 – Major Histocompatibility Complex, Class 1, C (HLA-C)
1.7 – Transmembrane Protein 194A (TMEM194A)
1.5 – Methyl-CPG Binding Domain Protein 3 (MBD3)
1.4 – Major Histocompatibility Complex, Class 1, G (HLA-G)

Cytoskeleton & Adhesion Molecules

4.1 – Desmoplakin (DSP)
3.7 – Dysferlin, Limb Girdle Muscular Dystrophy 2B (autosomal recessive) (DYS) †
3.0 – Laminin, β1 (LAMB1)
2.8 – Keratin 8 (KRT8)
2.5 – Golgi Apparatus Protein 1 (GLG1) † **
2.3 – Downregulated in ovarian cancer (DOC1) †
2.2 – Keratin 18 (KRT18)
2.1 – Absent in Melanoma 1 (AIM1)
2.0 – Mitochondrial Ribosomal Protein L13 & L (MRPL13) (MRPL)
1.9 – Crystallin, mu (CRYM)
1.9 – Ribosomal Proteins L3 & L6 (RPL3) (RPL6)
1.9 – Sarcoglycan (SGCG)
1.9 – Septin 8 (SEPT8)
1.8 – Calponin 3, Acidic (CNN3)
1.8 – Cadherin 3, Type 1, (placental) (CDH3)
1.8 – SATB1 Homeobox 1 (SATB1)
1.8 – Ribosomal Proteins L9, L12, S14 (RPL9) (RPL12) (RPS14)
1.7 – Ribosomal Proteins Large P0 & S3, S7, S10, S23 (RPLP0) (RPS3) (RPS7) (RPS10) (RPS23)
1.6 – Ribosomal Protein S15a (RPS15A)

Enzymes & Inhibitors

2.5 – 24-Dehydrocholesterol Reductase (DHCR24) **
2.3 – O-Linked N-Acetylglucosamine Transferase (OGT)
2.1 – HECT and WWE Domain Containing 1 (HUWE1)
2.0 – Glutathione-S-Transferase Ω (GSTO1) **
2.0 – Mesoderm Specific Transcript Homolog (MEST)
1.9 – Carboxypeptidase E (CPE)
1.9 – Argininosuccinate Synthetase 1 (ASS1)
1.8 – Phospholipase D2 (PLD2)
1.8 – Protein S (Alpha) (PROS1)
1.8 – Ribonuclease, RNASE A Family 9 (RNASE9)
1.8 – ATPASE, Na+/K+ Transporting, 2 Polypeptide (ATP1A1)
1.8 – Vaccinia Related Kinase 1(VRK1)
1.8 – Serpine Peptidase Inhibitor, Clade A, 5 (SERPINA5)
1.7 – Cardiomyopathy Associated 5 (CMYA5)
1.7 – Serpine Peptidase Inhibitor, Clade G, 1 (SERPING1)
1.6 – Sterol-C4-Methyl Oxidase-Like (SC4MOL) **
1.6 – Eukaryotic Translation Elongation Factor 1 β 2 (EEF1B2)
1.6 – Muscleblind (drosophila)-like (MBNL1)
1.5 –Transcription Factor 4 (TCF4)

LIGHT-SPECIFIC

Signaling Molecules

-5.0 – Cyclin A 1 (CCNA1)
-2.5 – Serine/Threonine Kinase 24 (STK24)
-2.3 – Cell Division Cycle 2 (CDC2) **
-2.3 – Interferon, alpha Inducible Protein 6 (IFI6) **
-2.2 – A Kinase (PRKA) Anchor Protein 12 (AKAP12)
-2.2 – CBP/P300-Interacting Transactivator with Glu/ Asp-rich carboxy-terminal 1 (CITED1) **
-2.0 – CDC28 Protein Kinase 2 (CKS2)
-2.0 – Guanine Nucleotide Binding Protein, δ3 (GNG3) **
-1.9 – Phospholipase C, δ 1 (PLCD1)
-1.8 – Cholecystokinin (CCK)
-1.8 – Sortilin 1 (SORT1)
-1.6 – Angio-Associated, Migratory Cell Protein (AAMP)
-1.6 – Purine-Rich Element Binding Protein B (PURB)
-1.6 – PRKC, Apoptosis, WT1, regulator (PAWR)

Cytoskeleton & Adhesion Molecules

-7.8 – Outer Dense Fiber of Sperm Tails 2 (ODF2) †
-7.7 – Protamine 1 (PRM1)
-4.4 – Calmegin (CLGN)
-4.0 – ‡ Claudin 11 (CLDN1)
-2.2 – Kinesin Family Member 11 (KIF11)
-2.0 – Budding Uninhibited by Benzimidazoles 1 Homolog beta (BUB1B)
-2.0 – ‡ Tripartite motif-containing 9 (TRIM9)
-1.7 – Contactin 2 (CNTN2)
-1.7 – Stathmin 1 (STMN1)
-1.6 – Tubulin, alpha 4A (TUBA4)

Enzyme & Inhibitors

-4.4 – Phosphodiesterase 3B, cGMP-Inhibited (PDE3B) †
-3.3 – Glycerol Kinase (GK)
-2.8 – Hydroxyacyl Glutathione Hydrolase (HAGHL)
-2.7 – ‡ mutS Homolog 4 (*E. Coli*) (MSH4)
-2.5 – Exonuclease NEF sp (RGD1305412)
-2.3 – Coagulation Factor C (PROC)
-2.3 – Solute carrier family 2 (facilitated glucose transporter), member 4 (SLC2A14)
-2.2 – Phosphorylase Kinase, γ 2 (testis) (PHKG2)
-1.8 – Serpine peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 (SERPINF1) **
-1.8 – Forkhead Box G 1 (FOXG1)
-1.8 – Signal peptidase complex subunit 1 homolog (SPCS1)

* Differentially expressed genes in dark and light testis tissues from at least 2 of the 3 horses are shown. Numbers represent balanced difference values for gene products considered differentially expressed.
** Indicates genes differentially expressed in both microarray experiments.
† Indicates genes differentially expressed in all 3 horses.
‡ Indicates average of only 2 usable balanced difference values.
Bold identifies genes further studied

An additional 88 genes appeared to be regulated differently between light and dark testis tissue in at least 2 of the 3 horses analyzed. These genes fall into a number of different functional categories, including 1) signaling molecules; 2) cytoskeletal and adhesion molecules; and 3) enzymes and inhibitors. These gene products and their official symbol (provided in parenthesis following gene names) are shown in Table 1 ranked by their balanced difference values averaged across all three prepubertal microarray analyses (number to the left of the gene name). Larger absolute values of balanced differences related to larger magnitudes of differential gene expression. For example, the largest (-7.8) was for ODF2, indicating that light testis tissue had 7.8-fold more ODF2 mRNA than the dark tissue of the testis. In total, 93 genes appeared to be differentially expressed based on microarray results. These included 58 genes preferentially expressed in dark testis tissue (left panel) and 35 genes preferentially expressed in light testis tissue (right panel). These 93 genes were only 1.3% of the 6940 gene products that had hybridization signals that met minimal criteria on at least 2 microarrays.

These microarray results indicated differential expression of many interesting genes. In the cell-signaling group, the insulin system was implicated, with insulin-like growth factor 2 and insulin-like growth factor binding protein 7 being predominantly expressed in dark tissue, whereas PDE3B was expressed more in light tissue. Gene products that regulate cell cycle progression were also differentially expressed. These included parathymosin and CDC like kinase in dark tissue and cyclin A1, CDC2,

CDC28 protein kinase 2, budding uninhibited by benzimidazoles 1 homolog beta, and PRKC, apoptosis, WT1, regulator (PAWR) in light tissue.

There were many differences in the expression of genes whose products had cytoskeletal or cell adhesion functions. For example, dark testis tissue expressed higher levels of desmoplakin, keratins, and cadherin mRNAs, the products of which are associated with intermediate filaments, as well as myosin-like DOC1, sarcoglycan, and calponin gene products, which are associated with large cytoskeletal filaments. Cell adhesion proteins laminin and GLG1 were also predominately expressed in dark testis tissue, as were two glyceraldehyde genes. Light testis expressed higher levels of gene products associated with sperm-specific structures (ODF2 and protamine 1) and microtubules (kinesin-like 1, stathmin, and the testis-specific tubulin A1). In addition, metabolic enzymes, kinases, protease inhibitors, and transcription factor genes were differentially expressed between dark and light tissues.

Study 1B analyzed the differences in gene expression in testicular parenchyma between an adult stallion with high fertility (designated as fertile stallion) and an adult stallion with reduced fertility (designated as subfertile stallion). Using similar criteria as in Study 1A with a ±1.7 balanced difference threshold value for genes to be differentially expressed, 233 gene products appeared to be differentially expressed. Of these, 122 genes appeared to be more highly expressed in the fertile stallion while 111 appeared more highly expressed in the subfertile stallion. Table 2 summarizes 30 of the genes found in the microarray study using a fertile stallion and a stallion with reduced fertility that met at least a 2.0 or greater minimum balanced difference. In this

Table 2. Differentially expressed genes in a fertile and subfertile stallion.*

FERTILE STALLION

Signaling Molecules

-2.4 - 5-Hydroxytryptamine (serotonin) Receptor 4 (HTR4)
-2.2 - Pre-B-cell Leukemia Homeobox Interacting Protein (PBXIP1)
-2.1 - Zinc Finger E-box Binding Homeobox 2 (ZEB2)
-2.0 - Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1 (CITED1) **
-2.0 - Forkhead Box O3 (FOXO3)
-2.0 - Low Density Lipoprotein Receptor-Related Protein Associated Protein 1(LRPAP1)
-2.0 - Interferon, alpha-Inducible Protein 6 (IFI6) **
-2.0 - Pre-B-Cell Leukemia Homeobox (PBX1)

Cytoskeleton & Adhesion Molecules

-2.6 - Selenium Binding Protein 1(SELENBP1)
-2.4 - ATP-Binding Cassette, sub-family F (GCN20), member 2 (ABCF2)
-2.3 - Plakophilin 4 (PKP4)
-2.1 - Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin) (SPTAN1)
-2.0 - Catenin (cadherin-associated protein), alpha-like 1 (CTNNAL1)
-2.0 - Catenin (cadherin-associated protein), beta 1, 88kD (CTNNB1)
-2.0 - Fibulin 2 (FBLN2)

Enzymes & Inhibitors

-4.9 - Rho-Associated, Coiled-Coil Containing Protein Kinase (ROCK1)
-2.9 - 24-Dehydrocholesterol Reductase (DHCR24) **
-2.5 - Cytochrome P450, Family 17, Subfamily A, Polypeptide (CYP17A1)
-2.1 - Serpine Peptidase Inhibitor, Clade F (alpha-2 antiplasmin, pigment epithelium derived factor), Member 1 (SERPINF1) **
-2.0 - Cytochrome P450, Family 11, Subfamily A, Polypeptide (CYP11A1)

SUBFERTILE STALLION

Signaling Molecules

2.0 - BTB (POZ) domain containing 9 (BTBD9)

Cytoskeleton & Adhesion Molecules

2.4 - Phosphatidylinositol Glycan, Class K (PIGK)
2.1 - Origin Recognition Complex, Subunit 6 like (ORC6L)
2.0 - Ankyrin Repeat Domain 36 (ANKRD36)
2.0 - DAZ Interacting Protein 1 (DZIP)
2.0 - Hypothetical Brain Protein MY040 (MY040)
2.0 - Stress-Associated Endoplasmic Reticulum Protein (SERP1)

Enzyme & Inhibitors

2.5 - Arginase, Type II (ARG2)
2.0 - ATP Synthase, H+ Transporting, Mitochondrial F0 Complex, Subunit B1 (ATP5F1)
2.0 - Telomerase Reverse Transcriptase (TERT)

* Gene products with a balanced difference value of ± 2.0 or greater are shown to the right of each gene name.

** Indicates genes differentially expressed in both microarray experiments.

Bold represents the two gene products with the highest balanced difference value in this experiment.

microarray, the two gene products with the highest differential expression values were Rho-associated, coiled-coil containing protein kinase, also known as ROCK1 (-4.9 in fertile tissue), and 24-dehydrocholesterol reductase (DHCR24), (-2.9 in fertile tissue). One of the genes that we further studied was StAR protein (not shown in Table 2) but was found to be more highly expressed in the fertile stallion (-1.7 in fertile tissue) compared to the subfertile stallion.

Additionally, results of the microarrays unveiled nine genes that were differentially expressed in both the light/dark and fertile/subfertile experiments (Appendix A). These genes included Golgi apparatus protein 1 (GLG1) (-1.8 in fertile tissue and 2.5 in dark tissue), glutathione-S-transferase (GSTO1) (-1.8 in fertile tissue and 2.0 in dark tissue), guanine nucleotide binding protein (GNG3) (-1.8 in fertile tissue and -2.0 in light tissue), cell division cycle 2 (CDC2) (1.7 in subfertile tissue and -2.3 in light tissue), serpine peptidase inhibitor, clad F, member 1 (SERPINF1) (-2.1 in fertile tissue and -1.8 in light tissue), sterol-C4-methyl oxidase-like (SC4MOL) (-1.7 in fertile tissue and 1.6 in dark tissue), 24-dehydrocholesterol reductase (DHCR24) (-2.9 in fertile tissue and 2.5 in dark tissue), interferon, alpha-inducible protein (IFI6) (-2.0 in fertile tissue and -2.3 in light tissue) and Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal, domain 1 (CITED1) (2.0 in fertile tissue and 2.2 in light tissue). Of the nine gene products expressed in both microarray experiments, four candidate genes (GNG3, SERPINF1, IFI6 and CITED1) were preferentially expressed in both the light parenchyma and in the fertile testis of stallions. These results suggest that these gene products could be potential markers of fertility in the stallion. GNG3 is a guanine

nucleotide-binding protein and is associated with the gamma subunit of G proteins which are involved in cell signaling (Hurowitz et al, 2000). Knockout mice for GNG3 demonstrate increased seizure activity and decreased body weight and overall adipose deposition when compared to controls (Schwindinger et al, 2004). SERPINF1 is an inhibitor of angiogenesis, also known as pigment epithelium-derived factor (PEDF), and is a member of the serine protease inhibitor family. Using knockout mice for PEDF as models, Doll and colleagues (2003) showed that fertility was unaffected and that treatment of prostate cells in culture with androgens inhibited the expression of PEDF. The functions of IFI6 and CITED1 are currently unknown and warrant further investigation as to their role in the testis. The other five gene products preferentially expressed in both microarrays included DHCR24, GSTO1, GLG1, SC4MOL, and CDC2. The functions of these gene products are challenging to understand because their balanced differential expression appears contradictive as they were found in dark parenchyma and fertile tissue (DHCR24, GSTO1, GLG1, and SC4MOL) or in light parenchyma and subfertile tissue (CDC2). DHCR24 is the most studied of this group and is an enzyme that converts sterols to cholesterol, a precursor to the production of steroid hormones (Wechsler et al, 2003). In mice lacking the DHCR24 gene, males showed normal organ development except in the testes which exhibited premature testicular degeneration rendering these mice infertile (Wechsler et al, 2003). GSTO1 is also an enzyme and has been reported to play a role in the detoxifying process of cells (Board et al, 2000). Little is known about the functions of GLG1, a protein associated with transport in the Golgi apparatus or with SC4MOL (Mourelatos et al, 1995). It

could be that these genes are initially involved lumen development of seminiferous tubules in dark testis tissue and later aid in the regulation of normal tubule maintenance in fertile stallions; however, more studies of these genes are needed. CDC2, found to be up-regulated in light parenchyma and subfertile tissue, is a cell cycle regulator involved with entry into mitosis and studies involving rats have shown that CDC2 induces apoptosis (Konishi et al, 2002). The up-regulation of CDC2 in light parenchyma of peripubertal stallions compared to dark parenchyma could be due to the presences of newly formed germ cells (namely spermatocytes and spermatids) found in spermatogenically active light tissue. The higher expression of the CDC2 gene product in subfertile tissue could be a result of low levels of cell division kinases (CDKs) which when present bind to CDCs giving the newly formed complex phosphoralytive properties (Lee et al, 1988). Collectively, these nine gene candidates need further investigation as they have the potential to provide important information about molecular functions in the stallion testis.

Northern blots confirm differential expression of DYS, DOC1, ODF2, and PDE3B genes in light and dark testes tissues

Northern blots were used to confirm the differential expression of DYS, DOC1, ODF2, and PDE3B genes between light and dark testis tissues, utilizing GAPDH mRNAs as a control. Representative Northern blots are depicted in Figure 1. In order to critically analyze and verify these results, horses evaluated were not used in the microarray. Both DYS (8.0 kb) and DOC1 (3.5 kb) mRNA concentrations are greater in dark testis tissue compared to light. Previous Northern blot experiments indicate similar

sizes for those mRNAs (Mok et al, 1994; Britton et al, 2000). In contrast, ODF2 and PDE3B mRNAs hybridized weakly in dark tissue but showed strong expression in the light tissue. Sizes of these transcripts (2.5 and 6.5 kb, respectively) were similar to those observed in other studies (Miki et al, 1996; Schalles et al, 1998). Similar Northern blot analyses of light and dark testis tissue RNA samples of four horses were quantitated. Since there were no differences in GAPDH mRNA concentrations found between light and dark tissues of other stallions in macroarray, *in situ* hybridization, or microarray analyses, each lane was normalized to its GAPDH mRNA signal and is reported as mean fold change for each mRNA (±SE). Fold differences on Northern blots were comparable to signal intensities for microarrays. Mean fold changes in dark versus light tissues for DYS and DOC1 mRNAs were 3.2 (±0.44) and 2.4 (±0.27), whereas ODF2 and PDE3B both exhibited fold increases in light over dark tissue at 4.6 (±1.6) and 3.9 (±1.2), respectively.

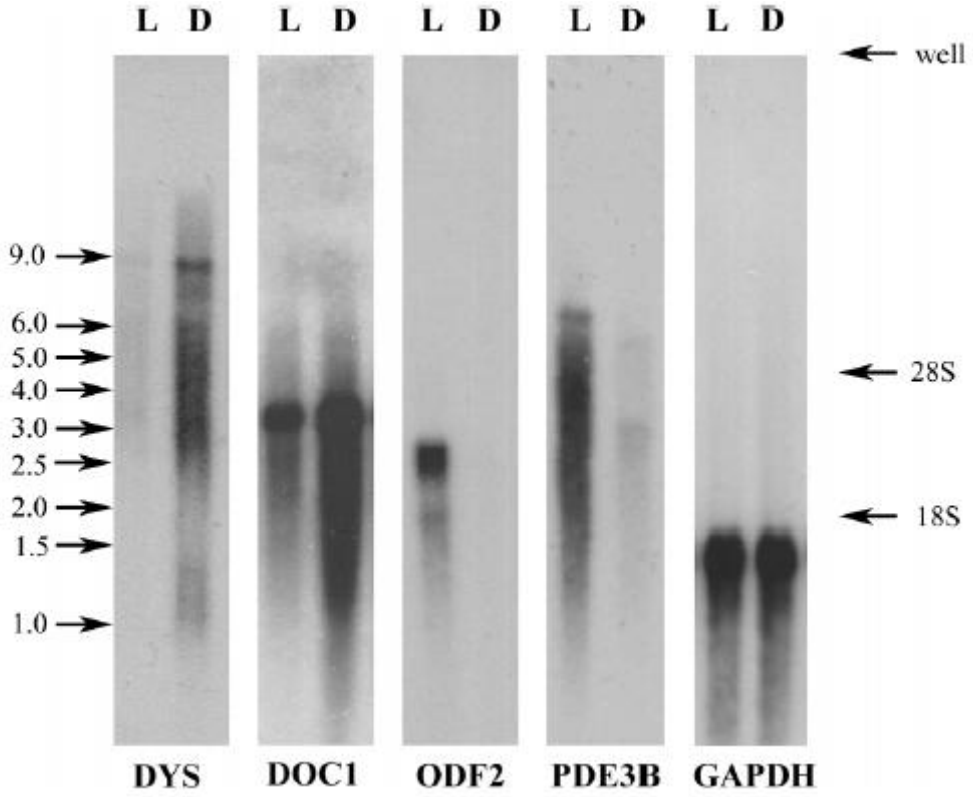


Figure 1. Representative Northern blot analyses of mRNA in dark and light regions of testes from 1.5-year-old horses. PolyA+ RNA was isolated from light (L) spermatogenically active and dark (D) spermatogenically inactive testicular tissues. Radiolabeled antisense cRNAs specifically hybridized dysferlin (DYS), down-regulated in ovarian cancer 1, myosin-like (DOC1), outer dense fiber of sperm tails 2 (ODF2), phosphodiesterase 3B (PDE3B), and 48lyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNAs on separate blots at 8.0-, 3.8-, 2.5-, 6.5-, and 1.8-kb sizes, respectively. Migration positions of RNA markers are indicated at left and ribosomal rRNAs at right.

In situ hybridization confirms and localizes differential expression of genes within dark and light testis tissues

DOC1, human DYS and PDE3B, and rat ODF2 cDNA localization of horse mRNA in light and dark testis from 1.5-year-old horses by *in situ* hybridization indicate similar patterns to those obtained by microarray and Northern blot analysis. Like that of the horse AR cDNA (GenBank accession number AY032721), the horse cDNA for DOC1 was highly conserved across species, being 92% identical to the human. Representative brightfield and darkfield views of the *in situ* hybridization of DYS and DOC1 mRNAs are shown in Figure 2. The bright-field views illustrate the histology of the testes; dark testis tissue has small seminiferous tubules with closed lumina and light tissue has larger seminiferous tubules and open lumina. Hybridization signals (small, black silver grains) for DYS and DOC1 mRNAs were markedly stronger in dark testis compared to the light tissue (Figure 2) and were concentrated over the seminiferous tubules. In contrast, an intense accumulation of signal for ODF2 and PDE3B mRNAs (Figure 3) was found in the light tissue over maturing germ cells (Figure 3). Thus, *in situ* hybridization confirmed the differential expression between dark and light testis tissue for four genes analyzed from the 93 genes listed in the Table and Northern blot hybridization.

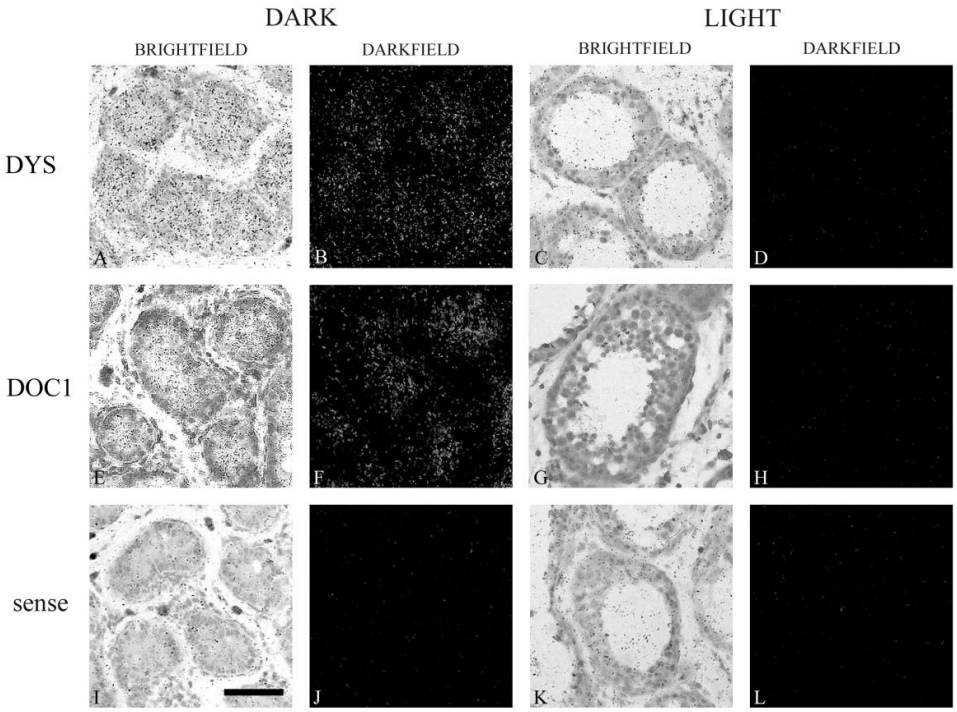


Figure 2. *In situ* hybridization reveals dysferlin (DYS) and down-regulated in ovarian cancer 1 (DOC1) gene expression predominantly in seminiferous tubules of the dark tissue of a maturing horse testis. Adjacent cross sections from a 1.5-year-old horse testis were hybridized with antisense cRNA probes for DYS and DOC1. Slides were coated with autoradiographic emulsion and developed after 2 or 4 weeks of exposure. Brightfield views (A, E, and I for dark tissue; C, G, and K for light tissue) show the nuclei stained lightly with 0.1% toluidine blue. Small silver grains shine white in darkfield views and indicate hybridization of radioactive probes for DYS (Panels B and D) and DOC1 (Panels F and H) mRNAs. Panels I-L show the low level of nonspecific binding to sense DYS cRNA (a negative control). They also clearly demonstrate the smaller diameter and lack of open lumina in the seminiferous tubules of the dark testis Panel compared to the light testis Panel. The bar in Panel I indicates 100 mm.

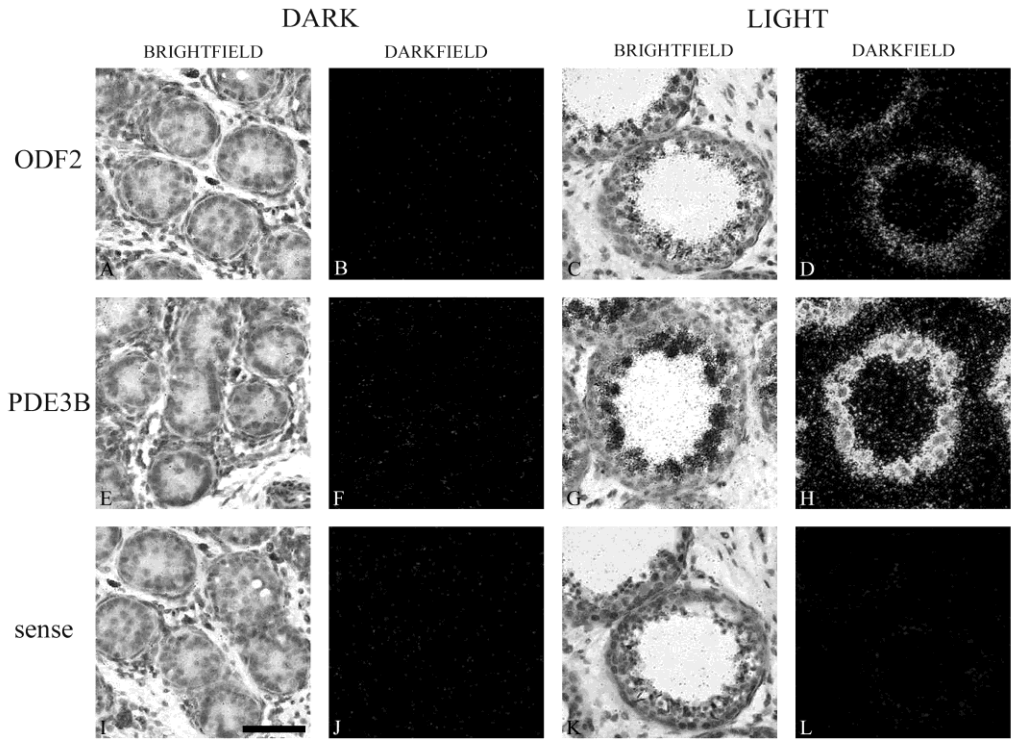


Figure 3. *In situ* hybridization localizes the expression of outer dense fiber of sperm tails 2 (ODF2) and phosphodiesterase 3B (PDE3B) mRNA in developing male germ cells. Representative views of adjacent cross sections of a testis hybridized with radiolabeled cRNA probes for ODF2 and PDE3B mRNAs. The ODF2 gene is primarily expressed in round and elongated spermatids (Panel D), whereas PDE3B mRNA demonstrates very high concentration in mature spermatocytes and round spermatids (Panel H). Tissue sections pictured in Panels I–L were hybridized to sense ODF2 cRNA (a negative control) and demonstrate the low level of nonspecific hybridization. The bar in Panel I indicates 100 mm.

Discussion

A major finding of these studies was that there are few changes in gene expression between light and dark testicular parenchyma or between a stallion of normal semen quality (fertile stallion) and a stallion with reduced semen quality (subfertile stallion) among the 9132 genes evaluated. The 93 genes identified by the microarrays in the light/dark study as being potentially differentially expressed comprised only 1.3% of those analyzed. Similarly, only 2.6% of the genes in the fertile/subfertile microarray were differentially expressed. In the light/dark microarrays, the use of matched tissues from the same horse testis provided a ‘‘low noise’’ model system that demonstrates how few changes in gene expression are concurrent with the initiation of spermatogenesis. Similar results on reproducibility and sensitivity of these types of microarrays have been reported (Yue et al, 2001; Moody et al, 2002; Reynolds, 2002).

The large number of gene products analyzed on the microarrays provided an unbiased and feasible method of identifying genes that are regulated during the initiation of spermatogenesis. The microarray data yielded expected results that validated the analyses. For example, AR and other genes analyzed previously were not differentially expressed between dark and light tissues of the horse testes. However, we were able to identify genes regulated during the initiation of spermatogenesis, including DYS, DOC1, ODF2, and PDE3B. Likewise, StAR protein and 24-dehydrocholesterol reductase (DHCR24) were found more highly expressed in the fertile stallion, as expected.

The DOC1 gene, a candidate tumor suppressor gene with a myosin-like product, was also found to be more highly expressed in dark tissue. Interestingly, DOC1 is

expressed by normal ovarian epithelial cells but not by ovarian cancer cell lines (Mok et al, 1994). Other than involvement with cytoskeletal and adhesion molecules like that of DYS, its role in the initiation of spermatogenesis is still unclear.

Expression of ODF2 and PDE3B genes is predominant in the spermatogenically active horse testis tissue and is described in other species, including the mouse, rat, and human (Hoyer-Fender et al, 1998; Wiersma et al, 1998; Peterson et al, 1999; Nakagawa et al, 2001). The ODF2 gene is expressed exclusively in the testis and is part of the cytoskeletal structure of the sperm tail. The function of ODF2 involves maintaining elastic recoil and providing protection from shear forces during sperm transport in the female reproductive tract. Equine ODF2 mRNA was localized to mature tubules with round and elongated spermatids, which coincides with the results of other studies in the rat and bull (Schalles et al, 1998). PDE3B also showed a definite expression difference in light tissue in all experiments conducted and functions to regulate cAMP levels. In the mammalian ovary, meiotic arrest of oocytes is regulated by cAMP levels. PDE3B enzymatically catabolizes cAMP levels, and when PDE3B is inhibited with products such as milrinone and cilostamide, cAMP levels increase and prohibit maturation (Wiersma et al, 1998). Also, PDE3B acts as a mediator in the insulin pathway (Harndahl et al, 2002). PDE3B mRNA was expressed only in secondary spermatocytes and round spermatids, but not in elongated spermatids. These results imply that PDE3B may play an important role in meiotic division in the testis. Other genes discovered by the microarrays that have greater expression levels in spermatogenically active light testis tissue specific to developing male germ cells include protamine, calmegin, tubulin a1,

and phosphorylase kinase γ2 (Amat et al, 1990; Prigent et al, 1996; Mochida et al, 1998; Yoshinaga et al, 1999; Steger et al, 2001).

Upon analysis, it is obvious that cross-species hybridization in cDNA microarrays is a strong tool to elucidate important genes involved in spermatogenesis. Although initial predictions of which hormone receptors and paracrine factors would be regulated during the initiation of spermatogenesis did not prove to be true, other genes were identified by the microarray analyses. One example is the CBP/p300 transactivator that participates in steroid hormone receptor and c-fos transactivation pathways and was preferentially expressed in light testis tissue (Fronsdal et al, 1998). Another example is the identification of three genes in the insulin-like growth factor–signaling system, which is consistent with the proposed role of this system in testis development and seasonal induction of spermatogenesis (Achanzar and Ward, 1997; Fiszer and Kurpisz, 1998; Liu et al, 1998; Wagoner et al, 2000).

Other groups have indicated that some of the gene products identified here are important to the biology of the testis (Yu et al, 2003). For example, ODF2, cholecystokinin, cyclin A1, calmegin, glucose transporters, and stathmin, predominately expressed in light testis tissues, are involved in meiosis and developing male germ cells (Persson et al, 1989; Weiss et al, 1997; Angulo et al, 1998; Flores et al, 1998; Ohsako et al, 1998; Rouiller- Fabre et al, 1998; Schalles et al, 1998; Bushby, 1999; Ravnik and Wolgemuth, 1999; Yoshinaga et al, 1999; Doege et al, 2000; Muller et al, 2000; Tsuruta et al, 2000; Guillaume et al, 2001). Additionally, placental cadherin and laminin are dark testis tissue–specific gene products that others have localized to peritubular cells and the

basement membranes of seminiferous tubules, respectively (Virtanen et al, 1997; Johnson et al, 2000a). Importantly, altered expression of DYS, kinesin, laminin, and protamine genes has been associated with reductions in male fertility and spermatogenesis in man and other species (Boekelheide et al, 1989; Lee et al, 1995; Virtanen et al, 1997; Mochida et al, 1998; Yoshinaga et al, 1999; Cho et al, 2001; Steger et al, 2001).

The use of human cDNA microarrays to study gene expression in the horse testis was highly successful. These Incyte microarrays have generated data in both human and animal systems that have proven to be reproducible and reliable (Pomp et al, 2001; Yue et al, 2001; Moody et al, 2002). The success of the technique in our studies is due to the high level of sequence conservation between horse and human mRNAs. Cross-species hybridization is common in Northern blot and *in situ* hybridization analyses, as demonstrated here even for species as diverse as rat and horse. Thus, microarrays composed of long human cDNAs (not oligonucleotides) provide a valuable tool for gene expression analyses in domestic animal species. Genetic analyses of equine testes were uniquely powerful in generating nonbiased lists of new gene targets to explore in the developing testis of the prepubertal colt and in stallions with normal and reduced semen quality. By understanding the regulation of the identified gene products within testicular cells of immature and mature stallions, we may elucidate the gene networks critical to efficient spermatogenesis as well as novel therapeutic approaches for improving male fertility.

CHAPTER IV

PDE3B AND StAR PROTEIN GENE EXPRESSION IN TESTES FROM NORMAL MATURE STALLIONS AND IMMATURE AND MATURE UNILATERAL CRYPTORCHID STALLION TESTES

Introduction

Spermatogenesis is the developmental progress of spermatogonia undergoing changes that yield highly specialized male gametes (Weinbauer and Wessels, 1999; Johnson et al, 2000b). Most likely, germ cells contain a specialized genetic program that controls the morphological changes. Steroidogenesis by Leydig cells in the testis is also required for spermatogenesis. One method of investigating specific genes involved in this progression is by *in situ* hybridization. Two transcripts, phosphodiesterase 3B (PDE3B) and steroidogenic acute regulatory protein (StAR) have been identified in this complex process in the testis (Reinhardt et al, 1995; Stocco and Clark, 1996; Ing et al, 2004). However, regulation of PDE3B and StAR protein gene expression has not been fully investigated in the stallion (*Equus caballus*). Therefore, the intent of this study was to evaluate gene expression differences in the testes of immature and mature horses and in unilateral cryptorchid horses. Unilateral cryptorchids, a congenital condition where one testis fails to descend into the scrotum, provide a unique model to study testicular function and dysfunction due to the presence of both a descended and retained organ. Additionally, we investigated potential relationships between these two genes and factors that influence fertility such as gonadal characteristics and circulating reproductive hormone levels in mature stallions.

Cyclic nucleotide phosphodiesterases are enzymes that hydrolyze cyclic nucleotides (Park et al, 2002). Of the 11 structurally-related family members, PDE3 is unique in its ability to terminate the action of both second messengers guanosine 3', 5'-cyclic monophosphate (cGMP) and adenosine 3', 5'-cyclic monophosphate (cAMP) (Loughney and Ferguson, 1996). Two members of the PDE3 family have been identified (PDE3A and PDE3B) and they have distinct genes located on different chromosomes. PDE3A maps to human chromosome 12 and PDE3B maps to human chromosome 11 (Miki et al, 1996). Northern blots have detected PDE3B mRNA in adipose and testicular tissues (Taira et al, 1993; Ing et al, 2004). Additionally, *in situ* hybridization has demonstrated high levels of expression in adipose tissue of developing rats and lower levels of expression in hepatocytes, oocytes, renal collecting duct epithelium and spermatocytes of mature animals (Reinhart et al, 1995). Levels of PDE3B mRNA increased with initiation of spermatogenesis in stallion testes (Ing et al, 2004). This observation suggests that PDE3B has a role in the regulation of spermatogenesis.

StAR protein transcripts in the testes have likewise been documented (Pollack et al, 1997; Manna et al, 2004; Manna and Stocco, 2005). StAR protein serves as a translocation enzyme necessary for cholesterol delivery to the inner mitochondrial membrane initiating steroidogenesis (Sugawara et al, 1995; Caron et al, 1997). Its necessity has been shown in the human condition called congenital lipoid adrenal hyperplasia (lipoid CAH). In patients with lipoid CAH, steroidogenesis is blocked as a result of a mutation in the synthesis StAR protein which results in the accumulation of

lipids (Stocco, 2002). Due to its key regulation of cholesterol trafficking, the temporal abundance of this enzyme may affect fertility directly associated with steroid production. These results have lead to our further investigation of StAR protein transcripts in the stallion testes.

The purpose of this study was to demonstrate PDE3B and StAR protein mRNA expression differences among mature stallions (testis weight > 150 gm) and unilateral, immature, one-year-old and mature, three-year-old cryptorchid stallions. Additionally, we sought to quantify the amount of PDE3B and StAR protein mRNA expression in the testis of mature stallions with the intent of measuring the strength of relationship between the amount of mRNA expression and fertility parameters such as daily sperm production (DSP), daily sperm output (DSO), apoptotic rate and circulating hormone levels. We hypothesized that: (1) *in situ* hybridization would reveal differences in gene expression for PDE3B and StAR protein mRNA in ten mature horses; (2) quantitative values from *in situ* hybridization experiments for PDE3B and StAR protein mRNAs would correlate positively with DSP and serum testosterone levels in those stallions; and (3) mRNA levels for PDE3B and StAR protein would be elevated in the descended testes of three-year-old unilateral cryptorchid stallions compared to one-year-old unilateral cryptorchid stallions and that the descended testes of both age groups would demonstrate a higher signal intensity compared to the retained testes.

Materials and Methods

Animals and tissue preparations

A total of twenty-two different stallion testicular tissue samples were used to evaluate gene expression changes of PDE3B and StAR protein mRNA by *in situ* hybridization methodology. Ten of these were archived left testis samples obtained from tissue blocks of mature stallions with normal-sized testes (>150 gm) of which measurements associated with fertility had been analyzed prior to castration. Of this population, horses were categorized based on semen quality parameters of progressively motile sperm (PMS) and morphologically normal sperm (MNS). Stallions were classified as normal with semen quality parameters >60% PMS and >55% MNS (*n*=9). One stallion (Peppy) was classified as having reduced semen quality because PMS was <23% and MNS was <39%. These samples were used to identify relationships between the estimated testicular amounts of PDE3B and StAR protein hybridization signal and parenchyma weight (g), DSP/gram of tissue, DSP/testis, DSP/horse, DSO, apoptotic rate, and circulating concentrations of testosterone (T), estradiol 17β (EEL), luteinizing hormone (LH), follicle stimulating hormone (FSH) and inhibin.

The remaining 12 tissue samples used in this study were whole testes from six unilateral cryptorchid stallions (three one-year-old stallions and three three-year-old stallions). These samples were harvested from client owned stallions admitted to the academic hospital (Large Animal Hospital at Texas A&M University, College Station, TX, USA) for elective castration. Castrations were performed by opened or closed surgical methods in lateral recumbence under general anesthesia, using anesthetic

regimens preferred by the surgeon and anesthesiologist on duty. These testes were used to determine differences in PDE3B and StAR protein mRNA concentrations between normally descended testes and retained testes. Immediately following tissue collection, each testis was weighed and testicular parenchyma (2 cm x 2 cm x 4 mm) was removed and fixed by 24 h immersion with rocking in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.2). Samples were then stored in 70% ethanol and embedded in Paraplast (Fisher Scientific, Houston, TX, USA) and sectioned for *in situ* hybridization studies.

Companion studies have previously described the methodology for determining DSO, DSP and apoptotic rates (Johnson and Thompson, 1986; Blanchard and Johnson, 1997; Heninger et al, 2004; Forrest et al, 2006). Briefly, DSO was estimated from the mean value of three daily ejaculate collections after seven prior daily ejaculates to clear stored sperm. DSP estimations were conducted by counting elongated spermatids following homogenization and DSP per testis was calculated from DSP/g and testis weight. The TUNEL (TdT-mediated dUTP Nick end Labeling) assay was used to calculate apoptosis in the testes and is expressed as the number of TUNEL-positive germ cells per 100 Sertoli cell nuclei (Heninger et al, 2006).

Hormone assays

Circulating serum levels of T, EEL, LH, FSH and inhibin concentrations were determined by radioimmunoassay (RIA) (Courtesy of J. F. Roser, Department of Animal Science, University of California, Davis, CA) for the ten mature horses used in this study. Heparinized blood samples were collected from the jugular vein once every hour

for four hours prior to sedation and castration. Plasma was isolated by centrifugation at 10,000 x g for 10 minutes and samples were pooled within stallions prior to conducting assay.

Cloning StAR cDNA

An equine probe for StAR protein mRNA was generated by cloning cDNA from the horse testes as previously described (Ing et al, 1996). PolyA+ RNA (200 ng) was reverse transcribed at 42°C with Superscript II (Life Technologies, Newark, NJ) and random hexamer primers. PCR was performed sequentially with two sets of primers designed to human cDNA sequence with the set of primers used for the second amplification nested inside the first. Outside primers were 5'-GCTCTTGGGCAGCCA CCCCT-3', 5'-AGTGATGAGTAAAGTGGTCCC-3' and inside primers were 5'-CCAAGCCTTCATTAACCCTCACTAAAGGGAGAGAGGTCGATGCTGAGTAGCC -3', 5'-CAGAGATGCATAATACGACTCACTATAGGGAGAAGATGTGGGCAAGG TGTTCCG-3'. PCR with outside primers and reverse-transcribed testis cDNA (20 cycles) was used as template for PCR with the inside primers (30 cycles). The cDNA was cloned into the PCR2.1 vector (Invitrogen, Austin, TX), and multiple clones were sequenced. The horse StAR protein sequence is in GenBank (accession number AY349170). The cDNA template for PDE3B antisense cRNA was a linearized plasmid previously described (Ing et al, 2004).

In situ hybridization

The mRNAs for PDE3B and StAR protein were localized on serial cross sections of testes by *in situ* hybridization to quantify expression as previously described (Ing et

al, 1997). Paraffin-embedded sections (5 µm) were mounted on glass Superfrost plus slides (VWR Scientific, West Chester, PA, USA) and sections hybridized at 42°C for 16 h with radiolabeled sense (negative control) and antisense PDE3B or StAR protein cRNA probes generated using *in vitro* transcription with α [^{35}S]-UTP (1250 Ci/mmol, New England Nuclear, Boston, MA, USA). A plasmid containing human PDE3B cDNA was restricted with *Bam*H1 and transcribed with T7 or T3 RNA polymerase for antisense and sense cRNAs. Antisense and sense horse StAR protein cRNAs were produced from PCR products with T3 and T7 RNA polymerases, respectively. Following hybridization, washing, and ribonuclease A digestion, slides were coated with photographic emulsion (Eastman Kodak, Rochester, NY, USA) and allowed to expose for 28 d. After emulsion development, cell nuclei were counterstained with 0.1% toluidine blue. Images were visualized using a Zeiss Axioplan 2 Microscope (Gottingen, Germany) and digital images were captured with a Nikon Alpha Photo Digital Imager and processed with Adobe Photoshop (Adobe Systems, Seattle, WA, USA).

Quantification of mRNA

Testicular expression of PDE3B and StAR protein mRNA was quantified for each stallion testis by measuring mean silver grain density (hybridization signal) on individual slides. Three randomly selected regions proximal to the central vein of each histological testis section were observed and captured under brightfield and darkfield microscopy. Five round seminiferous tubules from each of the three regions (n=15) were evaluated for PDE3B mRNA hybridization signal strength for each horse. StAR protein mRNA signal was evaluated similarly except the interstitial space where Leydig

cells reside was used for quantification. The image processing and analysis program, Scion Image Software (National Institutes of Health, Bethesda, MD, USA), was used for both PDE3B and StAR protein mRNA measurements. Scion Image was used to measure the mean silver grain density in selected regions based on a scale of 0 (white; representing hybridization signal) to 255 (black; representing a lack of hybridization signal) in darkfield images. Grayscale, pixel inversion and threshold parameters within the program were set prior to measurement analysis. Mean silver grain density was recorded for each testis by manually outlining round seminiferous tubules or interstitial space. Data were normalized by subtracting mean silver grain density acquired from sense control sections from antisense hybridized sections and by accounting for area of each measurement.

Statistics

Data for *in situ* hybridization quantification were analyzed by one-way ANOVA with means separated post hoc using Tukey's Honest Significant Difference (HSD) (SPSS© Inc., Chicago, IL, USA, 2001) and reported as mean silver grain density values in arbitrary units with standard deviation (SD) or standard error (SEM) where appropriate. Kendall's tau-b correlation coefficients were calculated for the analysis of relationships between individual horse testis, semen and hormone parameters, and either PDE3B or StAR protein mRNA mean silver grain density values. *P* values less than 0.05 indicated a significant difference.

Results

In situ hybridization identifies differences in gene expression for PDE3B and StAR protein mRNAs in testes of mature stallions

In situ hybridization was conducted on testicular tissues from ten mature stallions to determine differences in expression of PDE3B and StAR protein genes. Expression differences were apparent based on hybridization signals captured on images for both PDE3B and StAR protein mRNA (Fig. 4 and Fig 5, respectively). Distinct differences in signal intensity and transcript localization can be visualized in both the brightfield and darkfield views among the stallions. Figure 4 shows a noticeable difference in the expression of the mRNA for the PDE3B gene between the stallion with the highest intensity of signal (stallion 1957, panels A and B) and the stallion with the lowest intensity of signal (stallion Peppy, panels S and T). In addition, the PDE3B mRNA signal is specifically localized within the seminiferous tubules.

Likewise, Fig. 5 demonstrates gene expression differences among individual stallions for StAR protein mRNA. StAR protein has been found previously in the testicular Leydig cells, which are located in the interstitial space outside of the seminiferous tubules (Pollack et al, 1997; Kotula et al, 2001). Our data confirm these findings and demonstrates that hybridization signal intensity is different among individual stallions. One notable difference among hybridization signals of StAR protein was the total area occupied by the interstitial space. Stallions with higher hybrid-

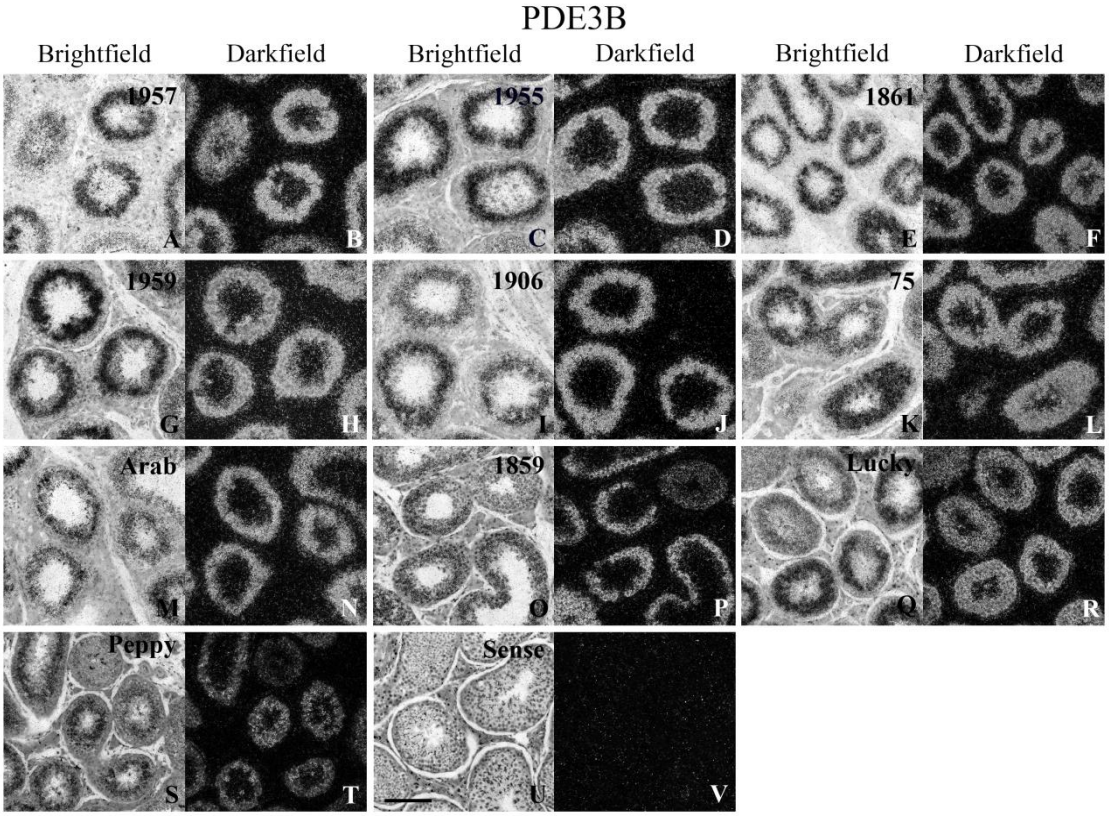


Figure 4. *In situ* hybridization reveals mean silver grain density differences of phosphodiesterase 3B (PDE3B) gene expression in seminiferous tubules of horse testis. Testis cross-sections were fixed with paraformaldehyde, mounted onto glass slides and hybridized with a PDE3B antisense cRNA ^{35}S-labeled probe. Cell nuclei were stained with 1% toluidine blue following autoradiography. Black silver grains on the brightfield images (panels A, C, E, G, I, K, M, O, Q, and S) and small silver grains displayed in white on darkfield images (panels B, D, F, H, J, L, N, P, R, and T) show representative images of *in situ* hybridization signals of PDE3B mRNA for ten horses. Non-specific binding to sense PDE3B cRNA (a negative control) is shown in panels U and V. The bar in panel U indicates 100 µm.

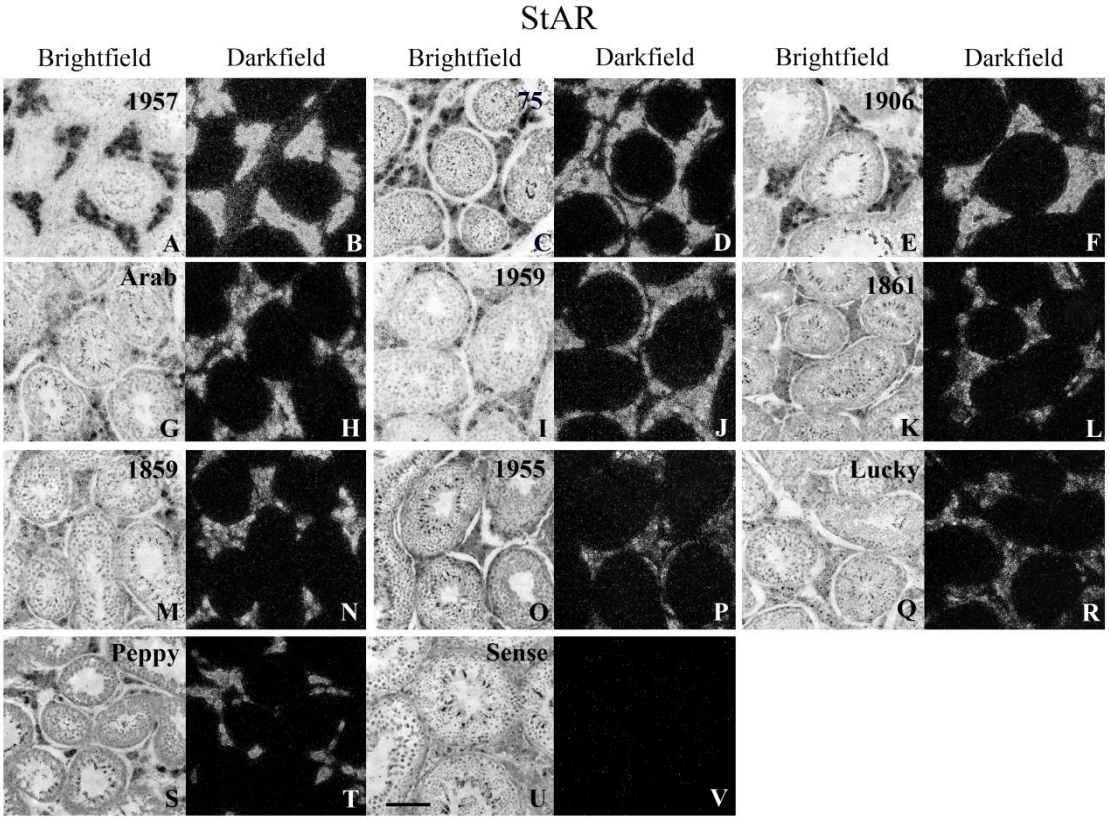


Figure 5. *In situ* hybridization shows differences in hybridization signal of steroidogenic acute regulatory protein (StAR) mRNA in the interstitial space of horse testis. Silver grain densities demonstrated on brightfield and darkfield slides are shown. Hybridization signal for StAR protein mRNA are localized in the interstitial space of the testis tissue. A noticeable decrease in the amount of signal (lack of white grains in darkfield panels) is observable between the stallion with the highest signal intensity (stallion 1957, panel B) and the stallion with the lowest signal intensity (stallion Peppy, panel T). Panels U and V represent sense images (negative control). The bar in panel U indicates 100 µm.

ization signal not only demonstrated a more intense signal suggesting more transcripts but the total signal area appeared to be greater than that observed for stallions with lower amounts of signal.

Quantitative in situ hybridization analysis reveals significant differences between horses for PDE3B and StAR

Mean silver grain density values (hybridization signal) from the *in situ* hybridization experiments were quantified by Scion image software and reported in arbitrary units (Table 3). One-way ANOVA was computed comparing the average hybridization signals for PDE3B among the ten mature stallions. A significant difference was found among the stallions for PDE3B ($P < 0.05$). Tukey's HSD was used to determine the nature of the differences between the stallions. The stallions (1957, 1955 and 1861) with the highest observed intensity of PDE3B mRNA were significantly ($m = 8.70$, $sd = 2.07$; $m = 8.66$, $sd = 1.01$; $m = 8.56$, $sd = 1.38$; respectively) higher and demonstrated over a two-fold increase in hybridization signal compared to the stallion (Peppy) with the weakest signal. Peppy ($m = 3.50$, $sd = 0.77$) was significantly lower in signal intensity than all other stallions. Similarly, quantitative data summarizing StAR protein is reported in Table 4. A significant difference ($P < .05$) was found among the stallions. This analysis revealed close to a three-fold difference in hybridization signal between the stallions (1957, 75 and 1906)) with the highest and the stallions (Peppy and Lucky) with the lowest values.

Table 3. Quantitative *in situ* hybridization results of phosphodiesterase 3B (PDE3B) mRNA and seminiferous tubule area in the testis of ten mature stallions. PDE3B mRNA data is expressed as mean silver grain density ± S.D. in arbitrary units (AU). The Area of seminiferous tubules is expressed as mean values ± S.D.

Stallions	PDE3B mRNA (AU)	Area of Seminiferous Tubules (AU)
1957	8.70±2.07[a]	5.02±0.83[a]
1955	8.66±1.01[a]	5.09±0.51[a]
1861	8.56±1.38[a, b]	4.64±0.76[a]
1959	7.47±1.18[a, b]	6.07±0.96[b]
1906	7.07±1.66[b, c]	6.23±1.02[b]
75	5.85±0.92[c, d]	5.35±0.40[a, b]
Arab	5.70±1.53[c, d]	6.30±1.40[b]
1859	5.44±1.20[d]	4.81±0.47[a]
Lucky	5.18±1.13[d]	5.59±0.78[a, b]
Peppy*	3.50±0.77[e]	4.80±0.56[a]

* Classified as having reduced quality semen (<23% PMS and < 39% MNS). All other stallions were classified as normal (≥60% PMS and ≥ 55% MNS) semen quality.

[a-e] Different superscripts within a column differ (P<0.05).

Table 4. Quantitative *in situ* hybridization results of steroidogenic acute regulatory protein (StAR) mRNA and measured interstitial space area in the testis of ten mature stallions. Data is expressed as mean silver grain density ± S.D. for StAR protein mRNA and mean ± S.D. interstitial space area in arbitrary units (AU).

Stallions	StAR mRNA (AU)	Area of Interstitial Space (AU)
1957	14.53 ± 1.93^{a}	$2.88 \pm 0.31^{a, b}$
75	14.49 ± 2.69^{a}	$3.00 \pm 0.40^{a, b}$
1906	14.41 ± 2.16^{a}	3.05 ± 0.39^{a}
Arab	$12.44 \pm 2.99^{a, b}$	$2.86 \pm 0.59^{a, b}$
1959	$11.48 \pm 2.20^{b, c}$	$2.69 \pm 0.40^{a, b}$
1861	$9.68 \pm 2.35^{c, d}$	$2.68 \pm 0.37^{a, b}$
1859	$8.40 \pm 2.01^{d, e}$	$3.00 \pm 0.63^{a, b}$
1955	$7.59 \pm 1.23^{e, f}$	3.01 ± 0.35^{a}
Lucky	$6.53 \pm 1.74^{e, f}$	2.51 ± 0.42^{b}
Peppy*	5.40 ± 1.23^{f}	1.94 ± 0.11^{c}

* Classified as having reduced quality semen (<23% PMS and < 39% MNS). All other stallions were classified as normal (≥60% PMS and ≥ 55% MNS) semen quality.

[a-f] Different superscripts within a column differ ($P<0.05$).

Correlations of PDE3B and StAR protein mRNA

The Kendall's tau-b correlation coefficient was calculated to determine the relationship between the gene expression values of PDE3B and StAR protein mRNA to testis, semen, and hormone levels of the ten mature stallions previously described. Mean (± SEM) values for parenchyma weight (g), DSP per gram, DSP per testis, DSP per horse, DSO, apoptosis rate, and circulating concentrations of T, EEL, LH, FSH and inhibin are summarized in Table 5 along with the calculated Kendall's tau-b correlation coefficient values for PDE3B and StAR protein mRNA (see Appendix B for individual stallion data). No significant relationships were found between PDE3B and testis, semen, or endocrine characteristics. However, a moderately positive correlation (r = .556) that was significant (p = .025) was found between StAR protein mRNA and testosterone. Although no other significant relationships were found for StAR protein mRNA, several other relationships were significant between gonadal characteristics and circulating hormone levels. Testosterone was positively correlated with parenchyma weight (r = .600, p = .015), DSP per testis (r = .555, p = .025), DSO (r = .555, p = .037) and negatively correlated with FSH (r = -.510, p = .040). Estradiol 17β was positively correlated with parenchyma weight (r = .510, p = .040) and negatively correlated with FSH (r = -.510, p = .040). Inhibin was positively correlated with parenchyma weight (r = .600, p = .016) and DSP per testis (r = .556, p = .025). Daily sperm per testis was positively correlated with parenchyma weight (r = .600, p = .016) and DSP per horse (r = .722, p = .007). Significant negative correlations were found between apoptotic rate

Table 5. Kendall's tau-b coefficients describing the relationship of mean values (± SEM) of equine testis, semen parameters, and circulating hormone concentrations to mean silver grain densities of phosphodiesterase 3B (PDE3B) and steroidogenic acute regulatory protein (StAR) mRNA quantified by *in situ* hybridization.

Gonadal characteristics and circulating hormone concentrations	*n*	Mean ± SEM	PDE3B (*r*)	Significance* (2-tailed)	StAR (*r*)	Significance* (2-tailed)
Testis and Semen Parameters						
Parenchyma Weight (g)	10	197.19±11.05	0.333	0.180	0.333	0.180
Daily Sperm Production (10^6)						
Per gram	10	15.59±0.64	-0.244	0.325	0.022	0.929
Per testis	10	3062±178	0.200	0.421	0.289	0.245
Per horse	9†	6228±375	-0.278	0.297	0.056	0.835
Daily Sperm Output (10^6)	9†	6356±472	-0.333	0.211	0.222	0.404
Apoptotic Rate	9††	7.47±1.13	0.167	0.532	-0.167	0.532
Endocrine Profile						
Testosterone (ng/ml)	10	1.40±0.16	0.200	0.421	0.556	0.025*
Estradiol 17β (pg/ml)	10	51.24±2.91	0.200	0.421	0.378	0.128
LH (ng/ml)	10	6.46±0.56	0.289	0.245	-0.067	0.788
FSH (ng/ml)	10	4.25±0.51	0.111	0.655	-0.160	0.531
Inhibin (ng/ml)	10	3.72±0.31	0.289	0.245	0.200	0.421

* Correlation is significant at the 0.05 level (2 tailed).

† Data for stallion 'Peppy' was not calculated.

†† Data for stallion 'Lucky' was not calculated.

and testosterone and inhibin (r = -.611, p = .022), DSP per testis (r = -.611, p = .022), DSP per horse (r = -.643, p = .026) and DSO (r = -.714, p = .013).

Gene expression changes in the testes of unilateral cryptorchid stallions

Expression of PDE3B and StAR protein genes was assessed by *in situ* hybridization and were studied in one-year-old (n = 3) and three-year-old (n = 3) unilateral cryptorchid stallion testes. PDE3B hybridization signal was highest in the three-year-old descended testes (Fig. 6; panel F). There were no observable differences in PDE3B gene expression between the three-year-old retained (panels G and H), one-year-old descended (panels A and B) or one-year-old retained (panels C and D) testes for PDE3B. All had very low hybridization signals. Figure 7 shows the *in situ* hybridization results for StAR protein mRNA in the testes of unilateral cryptorchid stallions. Differences are observed between the one-year-old representatives (panels B and D) where the abdominally retained testes demonstrate a stronger hybridization signal than the descended testes. As expected, the strongest StAR protein mRNA signals were found in the descended testes of the three-year-old horses (panels E and F). These appear to have intensities that are at least 2X greater than those in the retained testes of the three-year-olds (panels G and H). The weakest signal amongst the four groups is seen in the descended testes of the one-year-old horses (panels A and B).

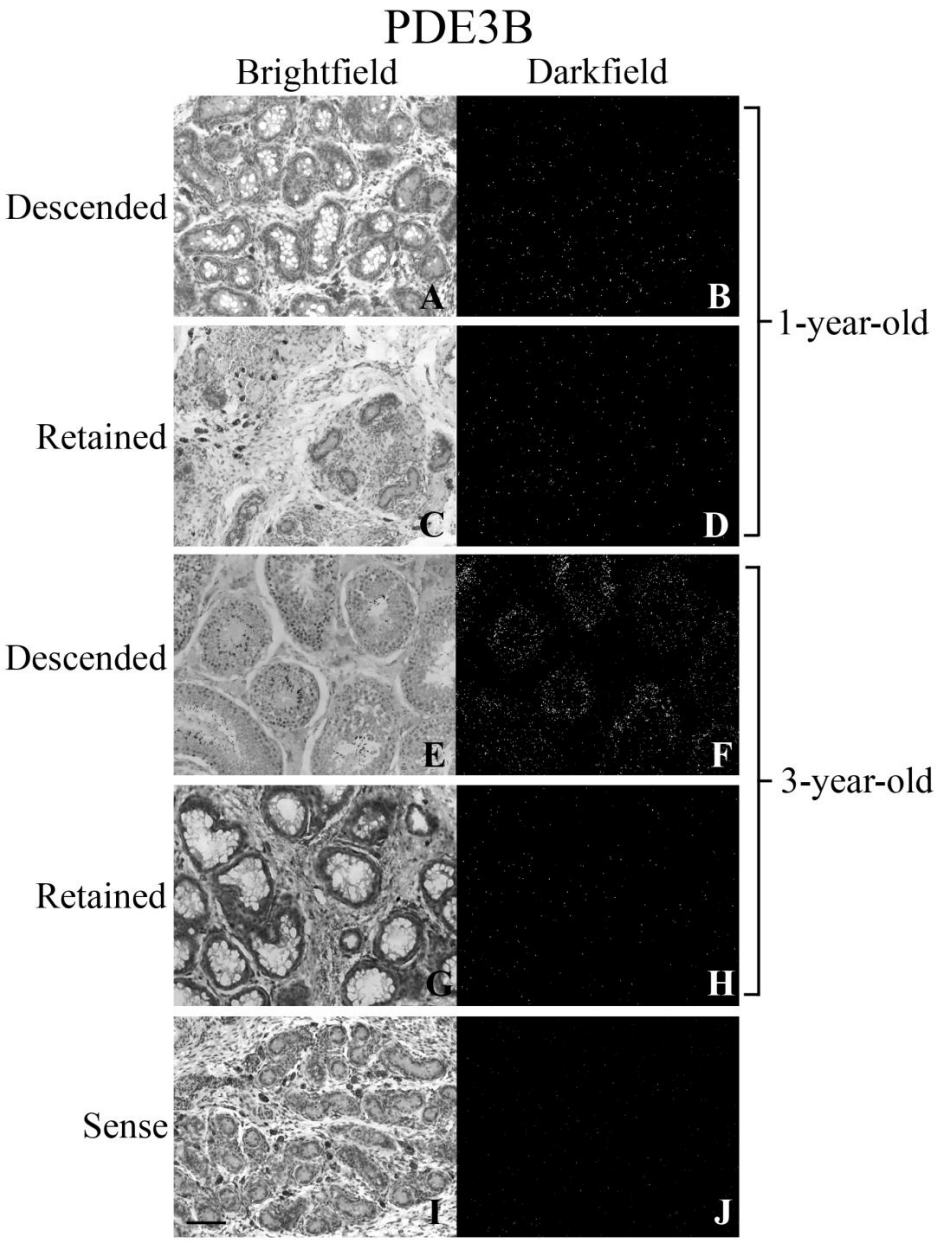


Figure 6. *In situ* hybridization of phosphodiesterase 3B (PDE3B) mRNA demonstrates differences in gene expression in seminiferous tubules of retained and descended testis of one-year-old and three-year-old unilateral cryptorchid horses. Matching brightfield (left column) and darkfield (right column) images show one-year-old descended testis (panels A and B), one-year-old retained testis (panels C and D), three-year-old descended testis (panels E and F) and three-year-old retained testis (panels G and H). Negative control sense PDE3B probe is shown in panels I and J. A difference in signal strength is visualized in the three-year-old descended image (panel F) compared to all others. The bar in panel I represents 50 µm.

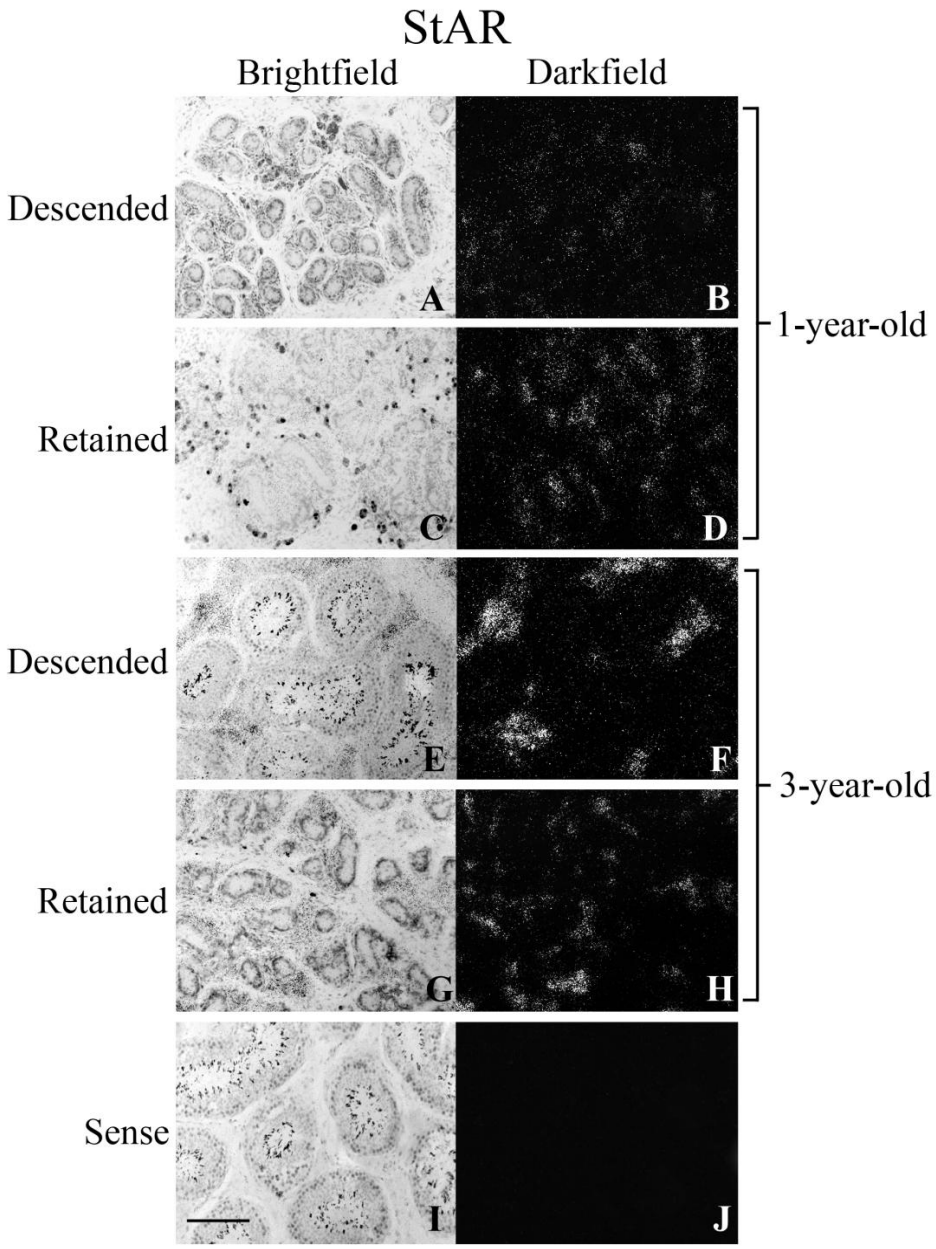


Figure 7. *In situ* hybridization reveals greater signal intensity for steroidogenic acute regulatory protein (StAR) mRNA in three-year-old descended horse testis compared to retained and descended testis of one-year-old and three-year-old retained testis representatives. Adjacent panels represent matching brightfield and darkfield images as one-year-old descended testis (A and B), one-year-old retained testis (C and D); three-year-old descended testis (E and F), three-year-old retained testis (G and H). A sense StAR RNA probe was uses as a negative control (panels I and J). Bar in panel I represent 50 µm.

Quantification of PDE3B and StAR protein mRNA reveals significant differences in unilateral cryptorchid stallion testes

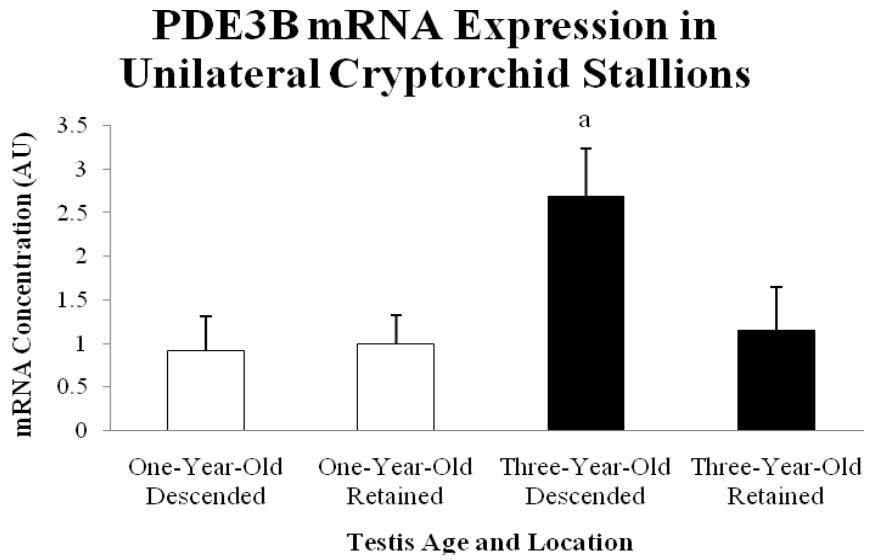
In order to more thoroughly investigate the observed differences found in the testes of unilateral cryptorchid stallions for PDE3B and StAR protein mRNAs, we quantified the hybridization signals and compared the differences by one-way ANOVA (Fig 8). A significant increase ($P < 0.05$) in the expression of the PDE3B gene (Fig. 8, A) was found in the three-year-old descended testes ($m = 2.68$, $sd = 0.55$) compared to all of the others. These data for PDE3B are consistent with our previous work and suggest that an increase in transcription of PDE3B mRNA occurs in spermatogenically active stallions (Ing et al, 2004).

Significant differences ($P < 0.05$) were also found for StAR protein gene expression (Fig. 8, B). The three-year-old descended testes had the highest hybridization signal strength ($m = 16.78$, $sd = 5.59$) and was significantly different from the three-year-old retained testes ($m = 9.15$, $sd = 3.87$). However, this trend was reversed in the younger stallions where the hybridization signal in the retained testes ($m = 10.20$, $sd = 2.91$) was significantly higher than the signal found in the descended testes ($m = 7.22$, $sd = 2.56$). No difference was found between the three-year-old retained testes and either of the one-year-old groups.

Discussion

In this study, *in situ* hybridization revealed differences in PDE3B gene expression in testes from ten mature stallions. PDE3B gene expression was highly specific to cells inside the seminiferous tubules and these findings complement previous

A.



B.

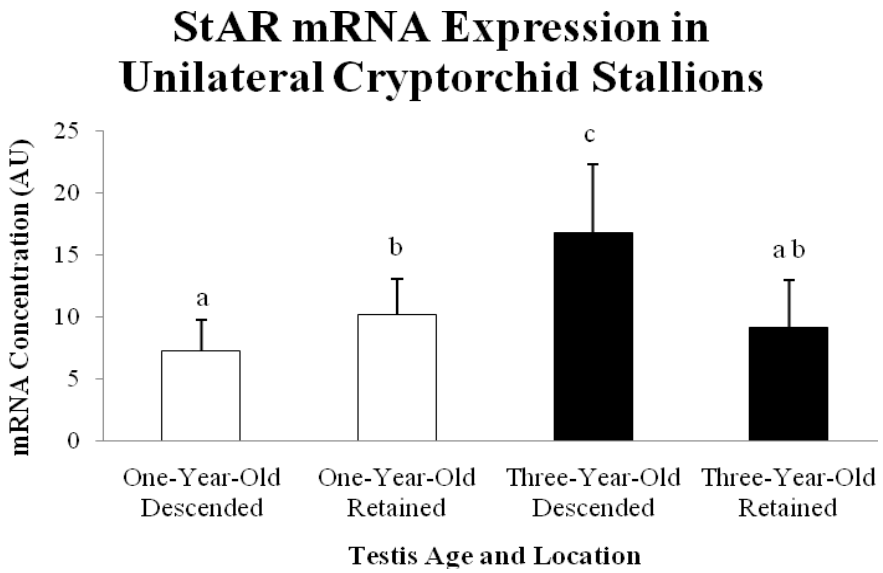


Figure 8. Quantification of phosphodiesterase 3B (PDE3B) (A) and steroidogenic acute regulatory protein (StAR) (B) transcripts in unilateral cryptorchid one-year-old (*n*=3) and three-year-old (*n*=3) stallion testes. Results are presented as mRNA levels calculated as mean silver grain densities from slides following *in situ* hybridization in arbitrary units. Background signal (pixel densities on sections probed with sense cRNAs) was subtracted from raw data and normalized to area of seminiferous tubules for PDE3B or interstitial space for StAR protein. Error bars represent standard deviations. Means with different superscripts differ ($P<0.05$).

work (Reinhart et al, 1995; Ing et al, 2004). However, this PDE3B gene expression was not localized to all spermatogenic cell types. This suggests that PDE3B is specific to certain germ cells, probably spermatocytes and more mature germ cells, because signal is not apparent in early spermatogonia cells that line the basement membrane. Sertoli cells cannot be excluded as possible candidates for producing PDE3B transcripts but our evidence suggests that they are not the dominant cell type for this mRNA because the hybridization signal does not span the entire area of both the basal and ad luminal compartments of the seminiferous tubule. Interestingly, strong hybridization signal for PDE3B (Fig 4, panels A and B) is visible in small ring-like, or circular patterns throughout the tubule. It was apparent that some tubules had less signal intensity than other adjacent tubules (not shown) which provides further evidence that the transcription of this gene may be specific to certain germ cells in the testis. It would be interesting in future studies to compare PDE3B signal strength in the stages of the seminiferous epithelium and to localize the signal to specific cell types. Significant differences in the amount of PDE3B mRNA were discovered among the 10 mature horses as initially hypothesized (Table 3). Of interest was the one horse categorized as having reduced quality semen also had the lowest concentration of PDE3B mRNA. In addition, the same horse also had the lowest value for parenchyma weight, DSP per testis and circulating concentrations of T, EEL, and inhibin and demonstrated one of the highest values for apoptotic rate compared to the other horses.

No significant correlations were found for PDE3B mRNA. Interestingly, there was a moderately negative, but not significant, relationship between PDE3B mRNA and

DSO (r = -.333, p = .211). This inverse relationship reveals that the lower amount of PDE3B mRNA may be related to horses with higher DSO and contradicts our original hypothesis that PDE3B would be positively correlated to DSO in stallions. In addition, the correlation coefficient for tubule area and DSO was close to a zero value (r = -.056, p = .835) and provides strong evidence that tubule area did not affect DSO in these stallions. However, additional work is needed to evaluate the relationship between the PDE3B gene and DSO as DSO, along with daily sperm production per horse and DSP per gram and was not calculated for the horse (Peppy) with reduced quality semen.

As predicted, *in situ* hybridization plainly showed gene expression differences among the mature stallion testes for StAR protein mRNA hybridization signal. As expected, the horse (Peppy) with poor quality semen and low circulating T also had the lowest signal intensity for StAR protein mRNA (Fig 5). Quantitation of StAR protein mRNA revealed differences between the highest and lowest values and approached a three-fold change in gene expression. Also, the method used to calculate gene expression for StAR protein mRNA demonstrated a difference between two horses (1861 and Peppy), where stallion '1861' had a significantly higher mean silver grain density than stallion 'Peppy'. StAR protein gene in these two horses was previously found in earlier experiments to be differentially expressed using microarray technology. These same results in different experiments help validate the use of the Scion Image Software program as a useful tool in investigating gene expression differences.

The number of Leydig cells per area was not evaluated in this study and would be valuable in future investigations. However, we can hypothesize that higher levels of

StAR protein mRNA signal corresponds to an increased density of Leydig cells in the interstitial areas in the testes. The moderately positive correlation found between StAR protein mRNA and testosterone indicate a significant relationship between the two variables and suggest that stallions with greater expression of the StAR protein gene tend to have a higher circulating amount of testosterone. To our knowledge, this is the first time this relationship has been reported in stallions or any other species. Also, our data for StAR protein mRNA and LH opposes other work that demonstrates an increase in StAR protein expression when ovine tissue was stimulated with LH (Price et al, 2000). One possible explanation for this discrepancy could be in the manner in which blood samples were taken to establish the endocrine profile. In this data set, one horse had a pooled mean value for LH three times higher than the mean value for all other horses. Additionally, it is possible that pooled samples did not adequately depict the dynamics of the hormone profile for each stallion.

Concentrations and localization of PDE3B and StAR protein mRNAs were evaluated in cryptorchid stallion testes. Gene expression differences were compared between one-year-old and three-year-old unilateral cryptorchid stallions and between descended and retained testes. An observable difference found by *in situ* hybridization was that PDE3B mRNA was only present in spermatogenically active tissue (the descended testes of the three-year-old post-pubertal horses). This agrees with our previous work (Ing et al, 2004). It has been reported that development of the germinal epithelium in retained testes is arrested and, therefore, the retained testes are not spermatogenically active (Coryn et al, 1981). Later studies described differences

between abdominally retained and inguinal retained testes where abdominal testis development did not proceed beyond B spermatogonia (equivalent to spermatogenesis in a four-month-old foal) and inguinal testis demonstrated some development up to early spermatocytes (equivalent to spermatogenesis in a 12-month-old foal) (Arighi et al, 1987; Al-Bagdadi et al, 1991). In this study, inguinal or abdominal retention was not documented at the time of testes harvest. Additional studies comparing the differences among descended, inguinally retained and abdominally retained stallion testes could provide further insight into the steroid hormone biosynthesis and intercellular communication in the equine testes.

Evaluation of StAR protein mRNA in the cryptorchids revealed interesting differences between the two age groups. In the three-year-olds, stronger hybridization signals were evident in the descended testes, while one-year-olds revealed stronger signal in the retained testes. To our knowledge, these age differences for StAR protein mRNA expression in unilateral cryptorchids are reported here for the first time. It may be possible to explain these differences solely based on Leydig cell numbers in the interstitial space, however, controversies exist about Leydig cell populations in unilateral cryptorchids and conclusive evidence has not been reported. Some authors report no differences between Leydig cell numbers in retained and descended testes of human patients (Sirvent et al, 1989). Others report that androgen production increases in tissues from unilateral cryptorchid rat testis when treated with LH compared to the contralateral testis and descended testes of normal animals (Jansz and Pomerantz, 1986). This study suggests that Leydig cell numbers in cryptorchid tissues may be elevated; however,

Leydig cell numbers were not reported. Clemmons (1995) indicated that there was an increase in Leydig cell numbers in spermatogenically inactive tissue (dark parenchyma) compared to spermatogenically active tissue (light parenchyma) of stallion testis. This increase was most likely due to smaller seminiferous tubules in the dark parenchyma which allowed for greater Leydig cell concentrations in the interstitial space. As the testis became spermatogenically active, seminiferous tubules increased in circumference and the total area of interstitial space decreased; therefore, decreasing the density of the Leydig cells. Johnson and Neaves (1981) also describe an increase in Leydig cell numbers as stallions increase in age; however, neither of the later two studies investigated cryptorchid testis.

In conclusion, we have shown gene expression changes by *in situ* hybridization in testes from mature normal and immature and mature cryptorchid stallions for concentrations of both PDE3B and StAR protein mRNAs. These results indicate that these genes are regulated in the testes. To date, most of the work with PDE3B has been focused on investigating its function and usefulness as a target for various drugs in adipose tissue, liver, pancreas and cardiovascular tissue, but not in the testis (Shakur et al, 2001; Bender and Beavo, 2006). Identifying the specific germ cells that transcribe the PDE3B gene requires additional investigation and would be beneficial in understanding the role of PDE3B in gamete development. Additionally, the findings of increased levels of StAR protein mRNA expression in three-year-old unilateral cryptorchid testes compared to one-year-old unilateral cryptorchid testes and that retained testes of one-year-old cryptorchids demonstrated significantly higher signal

intensity for StAR protein mRNA compared to one-year-old descended testes in this study may help us to understand the development of steroidogenesis.

CHAPTER V

IN VITRO CULTURE OF PRECISION-CUT TESTICULAR TISSUE AS A NOVEL TOOL FOR THE STUDY OF RESPONSES TO LH*

Introduction

Investigating the molecular functions of the testis has been challenging due to the many cytological interactions that occur within the organ. Development of *in vitro* culture techniques that support cellular survival and spermatogenesis will aid in our understanding of this complex tissue. Various methods have been used to elucidate regulatory mechanisms within the testis such as cell and tissue culture systems, but they have not been entirely successful (Staub 2001; Parks et al, 2003). Theoretically, organ culture systems are advantageous due to their controlled environments and the fact that they allow typical cellular interactions to proceed. However, their use has proven problematic due to an inability to maintain cell viability for extended periods of time and the need to supply uniform access of medium and gas exchange to the tissues (Orth et al, 1998).

Precision-cut tissue slice (PCTS) *in vitro* culture has emerged as a possible system to evaluate testicular functions. The precision-cut technique utilizes slivers of whole organs placed on wire mesh grids and incubated in small culture vessels containing medium and gases (Smith et al, 1985). This organ culture approach has

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provided a valuable means for investigation due to the reproducibility of mechanical tissue slicing, the ability to use organ slices that mimic tissues *in vivo*, and the maintenance of tissue architecture (Parrish et al, 1995). The PCTS *in vitro* culture system has been used successfully with several tissues such as liver, kidney, lung and heart (Parrish et al, 1995; Turney et al, 1999). Furthermore, PCTS has been used to challenge tissues *in vitro* from several animal models. These studies have included *in vitro* challenges with various compounds on mouse liver slices, rabbit renal slices and human prostate slices (Parrish et al, 1999b; Turney et al, 1999; Schmelz et al, 2001; Parrish et al, 2002). Although similar systems (Steinberger and Klinefelter, 1993; Orth et al, 1998) have been used to investigate somatic/germ cell interactions and gene expression in culture, to our knowledge this is the first report describing the use of PCTS with any equine tissue.

The purpose of the present study was to validate a short-term *in vitro* culture system using PCTS from stallion testes. Our approach was to challenge the equine testicular tissue with ovine luteinizing hormone (oLH) and measure the effects on 1) steroid hormone production and release into the medium and 2) mRNA gene expression. Following the 6-h *in vitro* culture, medium was collected and evaluated for testosterone (T) and estradiol-17β (E_2) concentrations. Additionally, the relative expression of steroidogenic acute regulatory protein (StAR protein), phosphodiesterase 3B (PDE3B) and outer dense fiber of sperm tails 2 (ODF2) mRNAs were quantitated. These genes were selected for analysis because of their past association with either initiation of spermatogenesis (Ing et al, 2004) or fertility status (not shown). Studies have also

shown that LH-induced StAR protein increases steroidogenesis in MA-10 mouse Leydig cells and that cells in culture have maintained viability based on production and release of T and E_2 into the medium (Clark et al, 1994; Manna et al, 2004).

In the current experiments, viability of the tissue during culture was assessed by evaluating histological integrity (histoarchitecture), measuring the release of lactate dehydrogenase (LDH), an intracellular enzyme, into the medium and steroidogenic activity (release of T and E_2 into the medium). We hypothesized that the administration of increasing doses of oLH would result in an increase in both T and E_2 production and secretion. Additionally, we hypothesized that mRNA levels of StAR protein would increase with increasing doses of oLH and that there would not be an observable increase in expression of PDE3B or ODF2 genes.

This study was designed to demonstrate that PCTS can be an effective tool for investigation of testicular tissue in stallions. Validation of this *in vitro* system will facilitate studies of mechanisms which regulate expression of genes that are involved in testis functions including spermatogenesis. Ultimately this system could be used to elucidate factors that impair or augment male fertility in all mammalian species.

Materials and Methods

Animals

Whole testes (84-133 gm, each) from three immature light breed stallions were collected post-mortem from a commercial abattoir. Immediately following organ harvest, testes were placed on ice and transported 2.5 h to Texas A&M University where they were weighed and decapsulated while chilled on ice.

Precision-cut tissue slices

Testicular slices were initially prepared by removing the distal ends of each testis and punching cylindrical cores (8 mm diameter) of tissue proximal to the central vein. Individual testicular slices were cut using a Brendel/Vitron Slicer (Tucson, AZ, USA) and held in oxygenated Krebs-Bicarbonate slicing buffer (pH 7.4, 4°C, O_2:CO_2, 95:5) following the procedures described by (Parrish et al, 1995). The overall mean slice weight and thickness were 13.85 ± 0.20 mg and 515.00 ± 2.33 µm, respectively. Fresh slices for incubation were aligned on titanium-mesh inserts (four slices per insert) and placed into scintillation vials with 1.7 ml of Dulbecco's Modified Eagles Media (DMEM) supplemented with 10% charcoal-stripped, heat-inactivated fetal bovine serum (FBS), glutamine (200 mM), penicillin/streptomycin (5,000 units/ml) and gentamycin (50 µg/ml) in an atmosphere of 95:5 O_2/CO_2. This system uses roller culture, where the vials rotate in the incubator and expose the slice surfaces to medium and gas throughout the time course of incubation. The roller system reduced tissue necrosis when compared with alternate incubation systems (Parrish et al, 1995).

Slice samples were pre-incubated in medium without oLH for 1 h at 32°C and fresh medium was replaced prior to challenge to allow cells to normalize following mechanical stress. Treatments consisted of medium containing 0, 5, 50 or 500 ng/ml of ovine luteinizing hormone (oLH; characterized by Dr. Parlow, NIADDK-oLH-25, purified from ovine pituitary glands, biological potency of 2.3 U/mg) to stimulate steroid hormone (T or E_2) production and release. Each testis was evaluated in triplicate cultures at each dose. Tissue slices were incubated with oLH for 6 h at 32°C. At the

conclusion of the incubation period, slices were removed from scintillation vials and random slices per treatment were weighed. The remaining slices were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), (pH 7.2) and embedded in Paraplast (Fisher Scientific, Houston, TX, USA). Slice thickness was measured after transverse cross-sectioning following paraffin embedding.

Cloning of StAR cDNA from horse testis

cDNA was cloned from the horse to make a high-specificity probe for StAR mRNA similar to cloning previously described (Ing et al, 1996). PolyA+ RNA (200 ng) from mature stallion testis was reverse transcribed at 42°C with Superscript II (Life Technologies, Carlsbad, CA, USA) and random hexamer primers. PCR was performed sequentially with two sets of primers designed to human cDNA sequence with the set of primers used for the second amplification nested inside the first. "Outside" primers were 5'-GCTCTTGGGCAGCCACCCCT-3', 5'-AGTGATGAGTAAAGTGGTCCC-3' and "inside" primers were 5'-CCAAGCCTTCATTAACCCTCACTAAAGGGAGAGAGGT CGATGCTGAGTAGCC-3',5'-CAGAGATGCATAATACGACTCACTATAGGGAGA AGATGTGGGCAAGGTGTCCG-3' for StAR protein cDNA. Inside primers had T7 or T3 promoter sequences on sense and antisense primers, respectively. The cDNA was cloned into the PCR2.1 vector (TA Cloning Kit, Invitrogen, Carlsbad, CA, USA), and multiple independent clones were sequenced. The horse StAR cDNA sequence was submitted to GenBank (accession number AY349170).

In situ hybridization

The mRNAs for StAR, PDE3B and ODF2 were localized by *in situ* hybridization

on serial cross sections of the equine testis slices generated from PCTS following the procedures of Ing et al. (1997). Paraffin-embedded sections were cut to a thickness of 5 µm and mounted on Superfrost Plus slides (VWR Scientific, West Chester, PA, USA). Tissue sections were hybridized at 42°C for 16 h with radiolabeled sense (control) and antisense cRNA probes generated by *in vitro* transcription with α [^{35}S]-UTP (1250 Ci/mmol, New England Nuclear, Boston, MA, USA). Antisense and sense horse StAR cRNAs were produced from PCR products with T3 and T7 RNA polymerases, respectively. Human PDE3B and rat ODF2 cDNA-bearing plasmids were restricted within the cDNAs with *Bam*H1 for PDE3B and *Nco*1 for ODF2 and transcribed with either T3 or T7 RNA polymerase, respectively, as previously described (Ing et al, 2004). Following hybridization, washing, and ribonuclease A digestion, slides were coated with photographic emulsion (Eastman Kodak, Rochester, NY, USA). After 28 d, slides were developed and cells were counterstained with 0.1% toluidine blue. Brightfield and darkfield images were visualized with a Zeiss Axioplan 2 Microscope (Carl Zeiss, Gottingen, Germany) mounted with an Axioplan high-resolution digital camera. Images were captured with Axiovision 3.0 software.

Quantification of mRNA signal

Testicular expression of StAR, PDE3B and ODF2 mRNA was quantified for each stallion by measuring hybridization signal strength (silver grains in darkfield measured by pixel density) on individual slides. Ten randomly selected round seminiferous tubules from representative slices were used for quantification of PDE3B and ODF2 mRNA. StAR mRNA signal was evaluated similarly except the interstitial

space was used for quantification. Each field was captured under brightfield and darkfield microscopy at 400 X. Scion Image Software (National Institutes of Health, Bethesda, MD, USA) was used to calculate pixel values in selected regions based on a scale of 0 (white; representing hybridization signal) to 255 (black; representing a lack of silver grains). Grayscale, pixel inversion and threshold parameters within the program were set prior to measurement analysis. Mean silver grain density was recorded for each testis by manually outlining round seminiferous tubules or interstitial space within the program. Data were normalized by subtracting background from control sections hybridized to sense cRNA.

Radioimmunoassay

At the conclusion of the incubation period, spent medium was collected from each sample, centrifuged to pellet cell debris and stored at -20°C (Catania et al, 2003). All spent medium samples were analyzed for T and E_2 using radioimmunoassay (RIA). For T determination, 250 µl of medium were extracted with 5 ml diethyl ether (EM Science, Cincinnati, OH, USA). Each sample was analyzed in duplicate and corrected for extraction efficiency of 71%. Standards were prepared by reconstitution in 500 µl of phosphate-buffered saline-gel buffer (PBSG) with known amounts of ^{3}H-testosterone (Perkin Elmer-New England Nuclear, Inc., Boston, MA, USA) and 100 µl of T (Colorado State University) ranging from 3.9 pg/ml to 8,000 pg/ml. Pooled bull plasma was used to calculate the intra-assay coefficient of variation (CV) and was 2.97%.

For E_2 determination, samples (30 µl) were extracted twice with 5 ml of diethyl ether and reconstituted in 600 µl of PBSG. Each sample was analyzed in duplicate. The

Ultra-Sensitive Estradiol RIA Kit DSL-4800 (Diagnostic Systems Lab, Inc., Webster, TX, USA) was used to determine hormone concentration in the medium by following the protocol provided. Extraction efficiency was 86% and the intra-assay CV was 4.46% calculated by using pooled pregnant ewe plasma.

Lactate dehydrogenase activity

Cell membrane integrity was determined by analyzing the supernatant medium for LDH release into medium using the TOX-7 LDH assay kit (Sigma, St. Louis, MO, USA) as previously described (Azri-Meehan et al, 1992; Parrish et al, 1999a; Catania et al, 2003). In this experiment, LDH was measured in the medium after 1 h and 6 h to assess the amount of damage that precision-cut slicing and incubation had on testis slices. Following overnight storage at 4°C, 120 µl of supernatant medium were combined with equal portion of LDH substrate, dye solution and enzyme in 96-well, flat-bottom plates in triplicate. Each plate was covered and allowed to incubate at room temperature (RT) for 20 minutes until the reaction was terminated with 12 µl of 1 N hydrochloric acid (HCl). Absorbance was measured with a microplate reader (Packard BS10000 Spectracount, Downers Grove, Il, USA) at a wavelength of 490 nm. Background optical absorbance was measured at 690 nm and was subtracted from primary measurements for each well (Decker and Lohmann-Matthes, 1988).

Histoarchitecture

A preliminary study was conducted to evaluate changes in tissue maintenance and cellular morphology and to determine the optimal incubation time of precision-cut testicular slices (see Fig. 9). In this initial time-course experiment, three mature (> 193

gm, each) equine testes were precision-sliced, as above, and incubated in medium alone at 32°C for 0, 3, 6, 12, 24 or 48 h time intervals. Additionally, in order to observe changes due to mechanical slicing, some tissue was not precision-cut and was used as a control. Sections (5 µm) from post-incubation testes slices on slides were stained with either hematoxylin and eosin (H&E) or 0.1% toluidine blue. Slides were visualized and representative images of round seminiferous tubules of each time interval were captured as previously described (Fig. 9).

Data analysis

Data are expressed as the mean ± standard error of the mean (SEM). All data obtained from RIAs were log-transformed to establish a normal distribution histogram. Assumptions for normal random distribution were met prior to analysis by one-way ANOVA (SPSS Inc., Chicago, IL, USA) for both hormone values and mRNA quantification. Tukey's post hoc test was used to separate means among groups with equal variances, whereas the Tamhane post hoc test corrected for unequal variances when present. The level of statistical significance was a $P \leq .05$.

Dose-response graphs for T and E_2 are presented as percent hormone where 0 ng of oLH was set at 100 percent and mean values of 5, 50 and 500 ng of oLH were divided by the 0 ng value and multiplied by 100. The data for mRNA quantification, (raw silver grain density values) were divided by area measured and reported as arbitrary units. Similar background values of the sense cRNA hybridization were subtracted.

Results

Cellular architecture of precision-cut slices after culture

Experiments were initially performed to evaluate changes in the maintenance of cellular morphology in precision-cut testis slices. Histologically, slice evaluations revealed maintenance of the testis tissue integrity after slicing and after culture for 0, 3 and 6 h (Fig. 9, panels A-D). Seminiferous tubule structure and interstitial cells appeared normal at these time points. Germ cells were properly organized into the basal and adluminal compartments. Spermatogonia and spermatocytes were distinguishable and tubules with open lumens and spermatids were visible at each time point. Myoid cells were apparent and provided distinction between the seminiferous epithelium and the interstitial space. Cells in the interstitial spaces appeared uniform. Although there was no change in tubular dimension or in amount of interstitial space observed after 12, 24 and 48 h of culture, germ cell organization in the seminiferous epithelium and lumen architecture gradually changed after 6 h of culture (Fig. 9). Germ cells began to migrate into the lumens of seminiferous tubules and germ cell organization markedly decreased. Vacuolization of spermatocytes and spermatids became more extensive and bi-nucleated cells were readily observed. At 24 h, the loss of germ cell organization was apparent. Multi-nucleated cells were observed along the basal compartment of the seminiferous tubules and lumen architecture was lost. Large nucleated cells were present where the lumen should be. Cell types within the tubules were not distinguishable at 48 h. At that

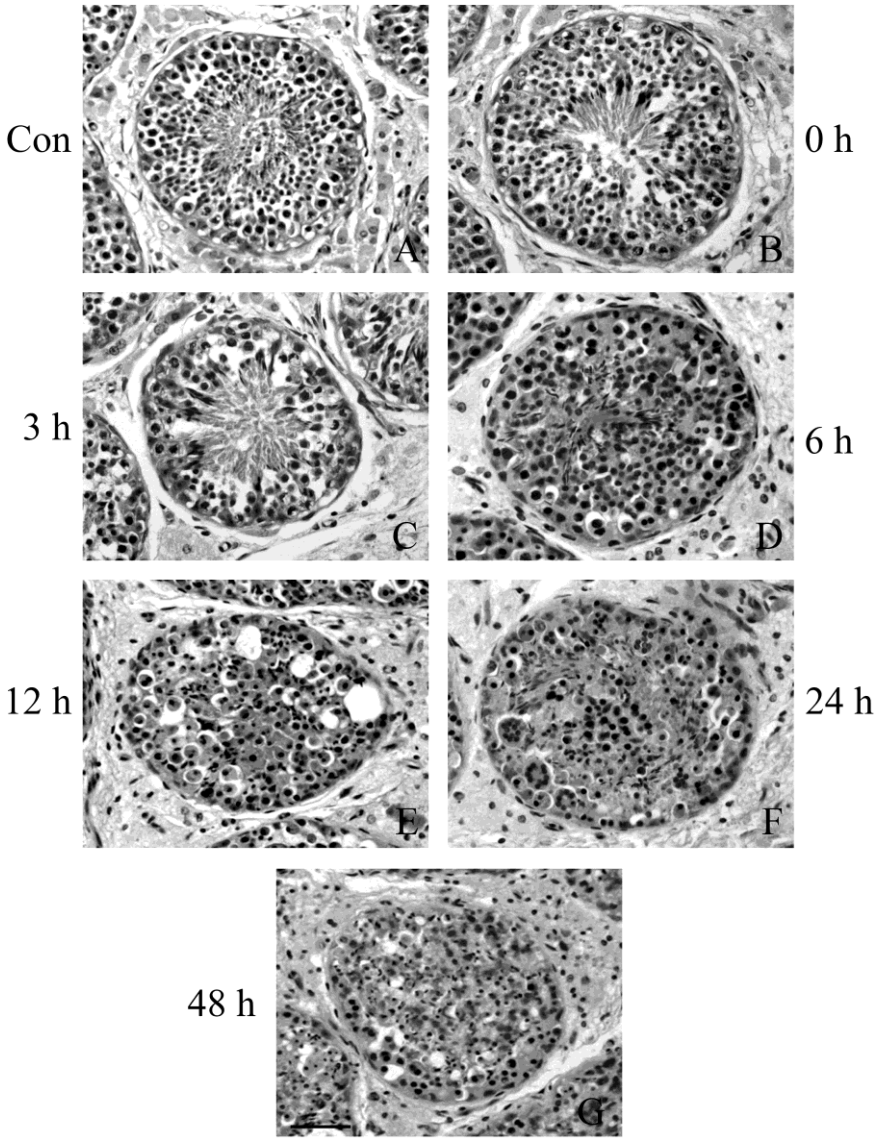

Figure 9. Histoarchitecture of precision-cut equine testis slices following culture. Mature testicular slices were placed into an *in vitro* organ culture system and maintained for 0 h (panel B), 3 h (panel C), 6 h (panel D), 12 h (panel E), 24 h (panel F) or 48 h (panel G). Following incubation, slices were fixed in paraformaldehyde and paraffin-embedded. Thin sections (5 µm) were cut, placed on glass slides and stained with hematoxylin and eosin. Con (panel A) represents tissue not precision-cut or cultured and serves as a reference for normal tissue integrity. The bar in panel G represents 50 µm.

time point, the lumen disappeared and the basement membrane began to break down which made it difficult to distinguish between the tubular epithelium and the interstitial space.

LDH secretion

Cell membrane integrity was assessed by lack of LDH release during culture experiments (Parrish et al, 1999a; Catania et al, 2003). No difference was found ($p > .05$) between 1 h and 6 h time points (data not shown). LDH was measured at 0 h (reference control) and a 1.47- and 1.53-fold increase over control was observed at 1 h and 6 h, respectively. These data demonstrate that tissue slicing did compromise plasma membrane integrity slightly, as expected, but 5 h of culture did not result in further damage.

Testosterone and estradiol synthesis and release in response to oLH

Precision-cut testis slices of testes from peri-pubertal stallions were cultured with either 0, 5, 50 or 500 ng of oLH for 6 h. Levels of T in medium represent androgen production and release by the tissue in culture. Figure 10 shows the percentage increase of T in medium with either 5, 50 or 500 ng of oLH compared to culture medium without (0 ng) the addition of oLH. The 0 ng oLH treatment represents basal steroid production and release by the testis tissue which averaged 1.12 pg of T per mg of tissue and was set to 100% in the graph. Addition of oLH increased the amount of T in the medium more than 400%. No significant differences were found between low, moderate and high doses. However, the moderate and high doses demonstrated numerically higher T concentrations than the lowest dose of oLH.

The E_2 response trend was similar to the T response in that all treatments with oLH increased production of E_2 at least 120% over the 0 ng oLH value (Fig. 10). Peak E_2 production was approximately 200% higher in the medium compared to the 0 ng oLH level. These data indicate that the testis tissue in culture was viable and responsive to hormone.

In situ hybridization localized and quantitated StAR, PDE3B and ODF2 mRNA

In situ hybridization was performed to localize and determine changes in the expression of StAR, PDE3B and ODF2 genes in precision-cut testis slices following a 6-h culture with or without oLH treatment. Hybridization signals for StAR protein mRNA (Fig. 11) revealed expression in the interstitial space of the testes within Leydig cells. Additionally, enhanced expression of StAR protein gene was observed in response to oLH challenges. An oLH concentration-dependent increase was observed for StAR protein mRNA. Quantitative data summarizing the *in situ* hybridization results are shown Figure 12. The *in situ* data are presented as percent change in gene expression compared to control (same testicular tissue; but without precision-cutting or culture). The weakest amount of signal was observed in the 0 ng oLH group (Fig. 12). The 5 ng and 50 ng oLH treatments both demonstrated StAR protein mRNA levels that were approximately 100% higher than the 0 ng group. In the 500 ng treatment group, the StAR protein mRNA hybridization signal strength was higher than other groups and approximately 170% greater than controls. Hybridization signals for PDE3B and ODF2 mRNAs were localized to the seminiferous tubules of the testicular parenchyma

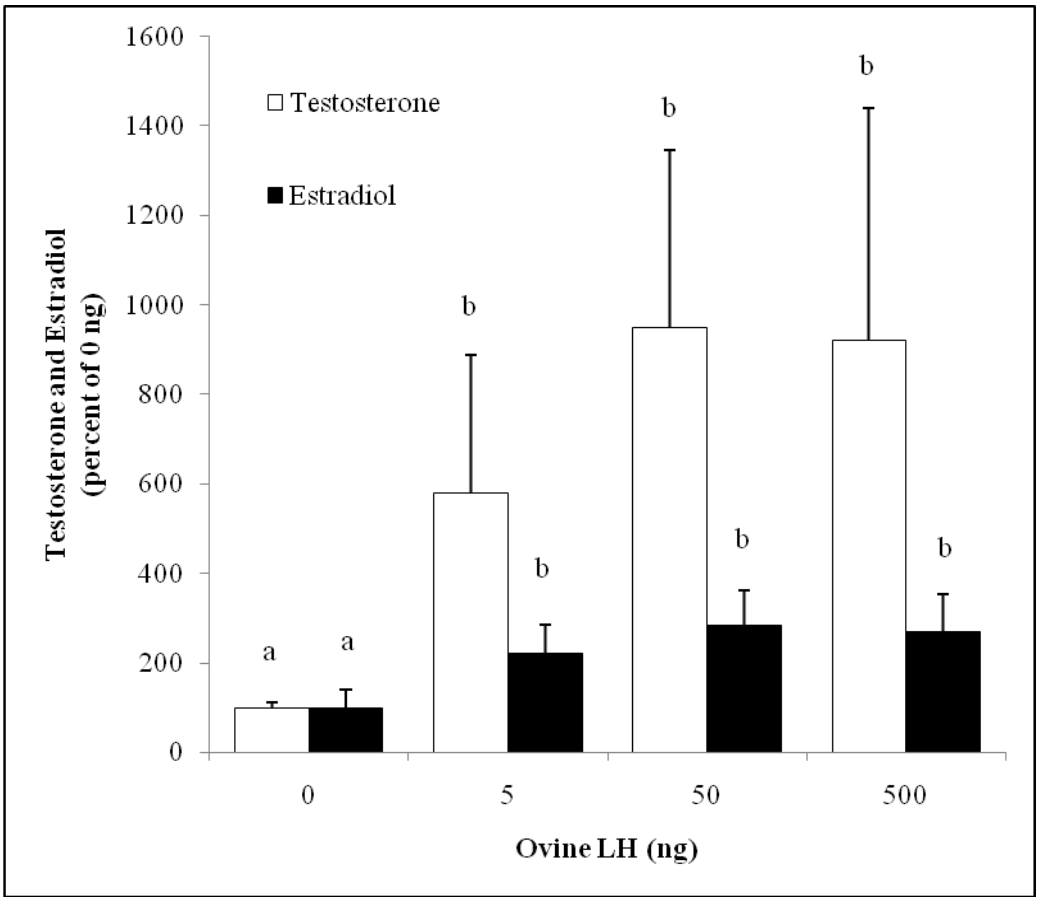

Figure 10. Percent testosterone (T) and estradiol (E_2) hormone production and release into medium by precision-cut equine testis slices following oLH challenge. Precision-cut slices were pre-incubated for 1 h prior to the addition of 0, 5, 50 or 500 ng oLH for 6 h. Data are the mean ± SEM of triplicate replications of three (n = 3) stallion testis and are expressed as the percent steroid produced at each treatment point where 0 ng of oLH was set to 100%. oLH stimulated an increase in both T and E_2 production over non-treated tissue (0 ng oLH). Means with different superscripts are different ($P < 0.05$) for T (white bars) and E_2 (black bars).

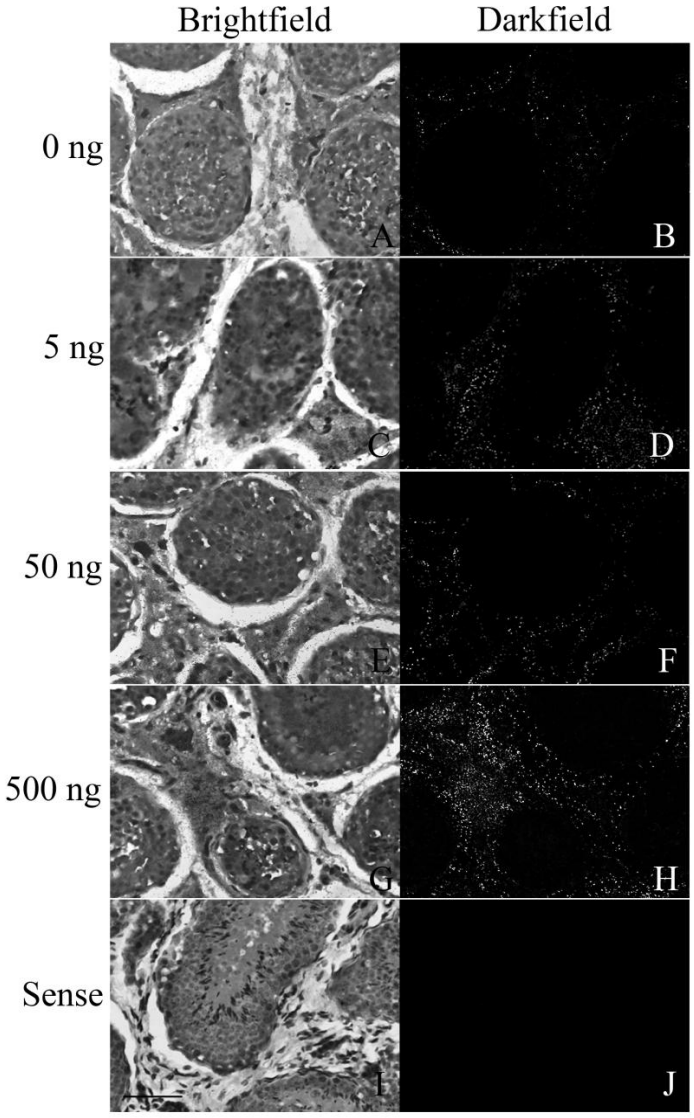

Figure 11. *In situ* hybridization of steroidogenic acute regulatory protein (StAR) mRNA levels in 6 h cultured precision-cut equine testis slices. Paraformaldehyde-fixed slice sections were mounted onto slides and hybridized with ^{35}S-labeled antisense StAR cRNA probe. Following autoradiography, cell nuclei were counterstained with 1% Toluidine blue. Black silver grains in the interstitial spaces on representative brightfield images (panels A, C, E, and G) show hybridization signals of StAR protein mRNA. Small silver grains shine white in darkfield images (panels B, D, F and H). Slices in medium were challenged with either 0 (panels A and B), 5 (panels C and D), 50 (panels E and F) or 500 ng of oLH (panels G and H). StAR protein mRNA expression increased with increasing doses of oLH. Panels I and J show the non-specific binding to sense StAR cRNA (a negative control). The bar in panel I represents 100 µm.

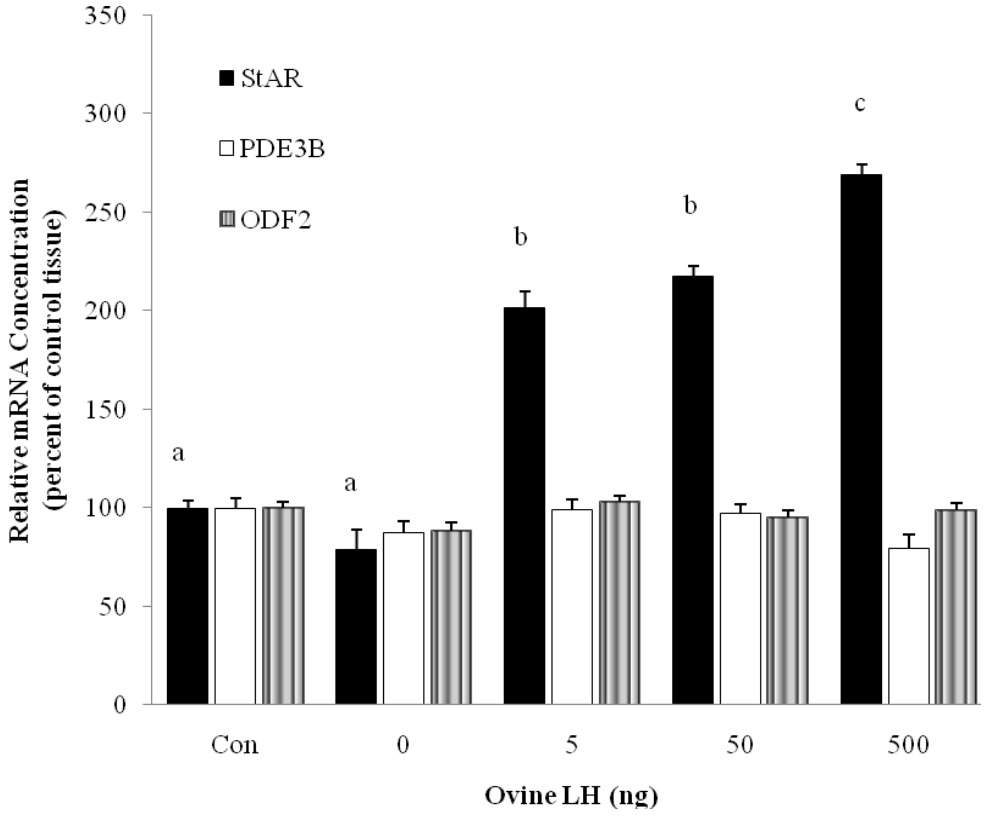

Figure 12. The percent change in gene expression for steroidogenic acute regulatory protein (StAR), phosphodiesterase 3B (PDE3B) and outer dense fiber of sperm tails 2 (ODF2) mRNA in precision-cut slices of equine testis tissue. Slices were incubated for 6 h in the presence of 0, 5, 50 or 500 ng of oLH. Control represents testicular tissue that was not precision-cut or cultured. Data represent the mean ± SEM silver grain density values quantitated by *in situ* hybridization and are shown as the percent mRNA gene expression quantified at each treatment level where control was set to 100%. Only StAR protein mRNA (black bars) was found to be different among treatments. Means with different superscripts are different ($P < 0.05$).

as expected (Ing et al, 2004). Although PDE3B and ODF2 mRNAs demonstrated strong hybridization signals, no increase in gene expression was observed in response to oLH treatments (Fig. 12).

Discussion

This study provides evidence that the precision-cut *in vitro* culture system is a useful experimental model for investigating hormone responses of the equine testis. These results demonstrate a system in which testicular tissue slices are maintained and responsive to oLH for at least 6 h in culture. The uniqueness of this *in vitro* culture system is that whole tissue, instead of dispersed cells, is used. In addition, the cultures are highly reproducible. One of the advantages of an *in vitro* system that utilizes precision-cut tissue is the consistent weight and thickness of each slice (Smith et al, 1985). Additionally, this system does not submerge the tissue continuously in the medium, but exposes the tissue to the medium and gas by floating the tissue on a grid in a continuously rotating culture vial (Parrish et al, 2002). The precision-cut tissue *in vitro* culture system has been used to study other tissues from several different animal models. However, this is the first report in which precision-cut tissue slices have been used to examine equine testis.

Similar incubation periods with other tissues have been described using the PCTS *in vitro* system and have even been extended successfully to 24 h (Parrish et al, 1999a; Schmelz et al, 2001; Catania et al, 2003). In our experiments, we used the 6 h incubation time based on our histological observations and that other equine testicular whole tissue culture systems used similar time points (Donnelly et al, 2007). Others

have cultured cell suspensions from equine testicular tissue for 12 h and 24 h (Eisenhauer and Roser, 1995; Hess and Roser, 2005). In rats, an organ culture utilizing 1-mm fragments of immature testicular tissue was maintained for 1 wk provided that FSH was added to the medium (Boitani et al, 1993). Our medium did not contain FSH. It might be possible to extend the incubation time without compromising the tissue by using a different media preparation or by including additional growth factors to the medium.

An important finding in this study was the establishment of viability in the testis slices during incubation. Viability was demonstrated by maintenance of histoarchitecture, the discovery that LDH leakage did not change from 1 h to 6 h, the increases in concentrations of T and E_2 in the media and StAR protein mRNA in the tissue in response to oLH challenges. In the stallion, T and E_2 production and release is regulated by equine LH. Equine Leydig cells treated with equine luteinizing hormone (eLH) demonstrate a dose-dependent increase of T and E_2 in the medium (Eisenhauer and Roser, 1995). Our findings were similar in that slices treated with oLH significantly increased their production and release of T and E_2 over untreated slices and indicated active steroidogenesis in the tissue and/or release by the tissue. However, the percentage increase in T and E_2 in response to oLH was not as dramatic in the testis slices compared to previously reported increases obtained in cultured Leydig cells (Eisenhauer and Roser, 1995; Hess and Roser, 2005). These differences may be explained by the different species of LH (ovine) used in these experiments and possibly the peripubertal stage of the stallions.

In situ hybridization results confirmed our hypothesis that expression of StAR protein mRNA would increase with increasing doses of oLH. It has been well documented that LH induces StAR protein gene expression (Clark et al, 1994; Stocco and Clark, 1996). Interestingly, one report showed a 1.4- and 3-fold increase in StAR expression in the testicular tissue of young and middle-aged rats, respectively, in response to LH treatment (Luo et al, 1998). Similarly, our data demonstrated an average 2.3-fold increase in stallion testicular tissue. Of the genes evaluated in this study, the role of StAR protein is the most understood in the testis. StAR protein is responsible for cholesterol delivery from the outer membrane of the mitochondria to the inner mitochondrial membrane enzyme cytochrome P450 cholesterol side chain cleavage (P450scc). This transport serves as the rate-limiting step in the production of steroids (Clark et al, 1994). StAR protein-null mice have a significant reduction in steroid production (Manna and Stocco, 2005). StAR protein transcripts have been localized in tissues that synthesize steroids including Leydig cells of testes, adrenal glands, ovary, placenta, and more recently the brain (Pollack et al, 1997; Manna and Stocco, 2005; Webber et al, 2006).

In conclusion, the precision-cut tissue slice *in vitro* culture system provides a novel tool to investigate equine testicular function. This system maintains whole tissue slices in a state where regulation of steroidogenesis and gene expression by cell stimuli can be explored. Future studies using this system could be useful in determining the effects of various exogenous compounds such as drugs on testicular tissue functions. Ultimately, this organ culture system could be used not only for reproductive studies in

stallions, but could be validated for use in a variety of species.

CHAPTER VI

CONCLUSIONS

The implementation of novel laboratory techniques, such as GIFT, ICSI, and oocyte transfer (OT) has greatly enhanced our knowledge of mammalian reproductive biology. However, as the field was concentrating on achieving high conception rates, little attention was paid to the causes of subfertility and infertility. As a result, the diagnosis and treatment of reproductive inefficiency has become a critical area of investigation in both humans and animals alike (Ehmcke and Schlatt, 2008). It has been reported that 15% of the human population is affected by some type of infertility/subfertility (De Krester and Baker, 1999). In farm animals, poor reproductive performance can lead to loss of income and genetic progress in populations due to failure to pass on viable gametes. Previous research has focused primarily on the female system; however, attention has shifted towards identifying the causative mechanisms in males. Approximately one-quarter of infertile/subfertile cases are male specific, and are identified as either irreversibly subfertile or having only limited treatment options (Swerdloff and Wang, 1993). References in the literature state that a subfertile male is an individual with abnormal semen quality and quantity which is reported to affect five percent of all men (Hirsh, 2003). This deficiency is also evident in the livestock species. Breeding stallions are especially prone to fertility problems, as evidenced by reports that approximately 38% of all prospective stallions fail a breeding soundness examination (Blanchard and Johnson, 1997). To date, there are few treatment options available once a male has been diagnosed as subfertile (Ball, 2008; Carnevale, 2008). Therefore, these

experiments were purposefully designed to investigate equine testicular genes and their role in regulating spermatogenesis and steroidogenesis. In addition, the knowledge gained by these investigations should expand the currently available therapies and enhance therapy development for the treatment of male infertility using the stallion as a model.

Two seminal articles initiated this project which discussed the differential shading of equine testicular parenchyma in peripubertal stallions and the possible gene differences between these two tissues (Clemmons et al, 1995; Johnson et al, 1997). Using the future research suggestions by these authors, we utilized microarray technology to evaluate gene expression differences in three equine peripubertal testes that exhibited both spermatogenically active (light testicular parenchyma) and spermatogenically inactive (dark testicular parenchyma) tissue in the same testicular samples. The microarrays evaluated over 9,000 gene products and the analysis determined that 93 genes were differentially expressed in at least two of the three horses. Of these, 58 were preferentially expressed in dark testis tissue and 35 were preferentially expressed in light testis tissue. The largest differential expression revealed was for the light specific ODF2 gene (-7.8) and was one of five genes discovered to be differentially expressed in all three horses. The other four included the dark specific genes DYS (3.7), GLG1 (2.5), and DOC1 (2.3) and the light specific gene PDE3B (-4.4). In addition, a second microarray was conducted which evaluated differential gene expression in the testes of a fertile and subfertile stallion. Using similar criteria, this microarray revealed 233 gene products that were differentially expressed. Of these, 122 genes were more

highly expressed in the fertile stallion while 111 appeared more highly expressed in the subfertile stallion. One of the gene products found to be more highly expressed was StAR protein mRNA which demonstrated a balanced difference value of -1.7 in fertile tissues.

Interestingly, results from the two different microarrays unveiled nine genes that were differentially expressed in both the light/dark and fertile/subfertile microarray experiments. Of these, four candidate genes (GNG3, SERPINF1, IFI6 and CITED1) were preferentially expressed in both the light parenchyma and in the fertile testis of stallions. These results suggest that these gene products could be potential markers of fertility in the stallion. The other five candidate gene products are more challenging to understand because their balanced differential expression was more evident in dark parenchyma and fertile tissue (DHCR24, GSTO1, GLG1, and SC4MOL) or in light parenchyma and subfertile tissue (CDC2). Currently, the literature provides some functional explanation for DHCR24, GSTO1, GLG1, and CDC2. DHCR24 is involved in the biosynthesis of cholesterol, GSTO1 is associated with cellular detoxification, GLG1 is a transport protein of the Golgi apparatus and CDC2 aids in the regulation of the cell cycle (Wechsler et al, 2003; Board et al, 2000; Mourelatos et al, 1995; Konishi et al, 2002). Limited information is available about SC4MOL, other than it seems to have enzymatic properties. Studies of these genes targeted at investigating differences in age of stallions, circulating hormone levels, events surrounding lumen formation in seminiferous tubules, and their expression in specific germ cells may help answer why these gene products seem to be expressed in uncommon testicular tissue. Overall, the

use of microarrays to study gene expression in the horse testis was highly successful revealing several candidate genes worthy of investigation. Continued investigations could potentially uncover the specific genes responsible for the current idiopathic nature of male infertility.

Some of the genes found by microarray experimentation were used for further investigation in to testicular function and regulation and included DYS, DOC1, ODF2, PDE3B and StAR protein. PDE3B and StAR protein, specifically, were evaluated by *in situ* hybridization to determine differences in mRNA expression in testicular tissues of mature stallions and mature and immature cryptorchid stallions. PDE3B was localized to the seminiferous tubules and significant differences in gene expression, determined by quantitating mean silver grain densities localized in the seminiferous tubules, were found among ten mature stallions. Interestingly, one stallion categorized as subfertile had a significantly lower mean value for PDE3B mRNA compared to the other nine stallions. We originally hypothesized that relationships would be found for PDE3B mRNA when compared to semen parameters, especially DSO, and circulating hormone values, specifically T, for the ten mature stallions. Although no significant correlations were revealed, our research demonstrated that additional investigations in the relationship between the expression of the PDE3B gene and DSO are necessary with more stallions. It would be beneficial to evaluate the relationship of PDE3B mRNA in multiple stallions demonstrating both high quality and poor quality semen characteristics. In addition, PDE3B mRNA was evaluated in one-year-old and three-year-old unilateral cryptorchid stallions. In this model, only the descended testes of the three-year-old stallions

demonstrated expression of the PDE3B gene. Future studies should investigate the expression of PDE3B mRNA in the seminiferous epithelium and correlate expression to tubule stage (Ing et al, 2004; Laughlin et al, 2009). Our results indicated that not all spermatogenic cell types express the PDE3B gene which suggests that it may be involved in the regulation of specific changes in spermatocytes and spermatids.

Likewise, StAR protein mRNA expression was evaluated by *in situ* hybridization and quantitated. Similar to PDE3B, StAR protein mRNA expression was different among the ten mature stallions. However, StAR protein mRNA was localized to the interstitial space surrounding the seminiferous tubules. We hypothesized that significant relationships between StAR protein gene expression and semen parameters such as DSP and DSO and circulating hormones, such as T and $E_{2,}$ would be revealed since StAR protein is responsible for cholesterol transport into the mitochondria. However, the only significant relationship found was between StAR protein and testosterone. One limitation to these correlative results is that DSP/horse, DSO, and apoptotic rate were not determined for all stallions used in this experiment. In the unilateral cryptorchid stallions, StAR protein gene expression was highest in the descended testes from three-year-old stallions when compared to the others, as expected. However, the retained testes of the one-year-old horses demonstrated a higher level of StAR protein mRNA expression when compared to their one-year-old descended testes. One research avenue that remains to be pursued is the correlation between StAR protein gene expression and number of Leydig cells per area. Our studies did not evaluate Leydig cell numbers in the tissues. However, this information could explain why immature retained testes exhibit

greater StAR protein gene expression than testes that have appropriately descended into the scrotum.

We have also demonstrated that one *in vitro* culture system is a valuable tool for investigating gene regulation mechanisms in testes tissues. Specifically, the precision-cut tissue slice (PCTS) *in vitro* culture system was utilized to evaluate its usefulness as a novel tool to investigate equine testicular function. The use of this system revealed that the slices produced by the precision-cutting technique were highly reproducible and were not different in weight or thickness. In this system, equine testicular tissue slices were incubated for six hours at 32°C. Although the slicing procedure did cause some damage to cells, LDH leakage did not change from one hour to six hours indicating the maintenance of the cell membranes. Precision-cut equine testicular slices were challenged with concentrations of 0, 5, 50 and 500 ng/ml of oLH and the tissue did respond by releasing T and E_2 into the medium during *in vitro* culture. The addition of oLH increased T and E_2 at least 400% and 120%, respectively, over the 0 ng control value. Testicular gene expression was also assessed with *in situ* hybridization methodology for StAR protein, PDE3B and ODF2 mRNAs. *In situ* hybridization revealed an oLH concentration-dependent increase in the concentration of StAR protein mRNA in Leydig cells. No differences were observed for expression of PDE3B or ODF2 genes in seminiferous tubules among treatment groups, as expected. These results demonstrate the value of *in vitro* culture of the precision-cut tissue slices for studies of steroidogenesis and gene expression in the stallion testes.

Future studies involving equine testicular tissue and PCTS should include attempts to extend incubation time without compromising the tissue and determining the effects of various exogenous compounds such as drugs on testicular tissue functions. In our medium, we did not include testosterone or FSH. Previous work has indicated that these two hormones may allow tissue to survive longer in culture (Boitani et al, 1993; Eisenhauer and Roser, 1995). One of the limitations to studying spermatogenesis using culture systems is that the complete process of spermatogenesis takes a long time, approximately 57 days (in the stallion), and we are currently only capable of incubating tissue for several hours at a time before normal testicular morphology is lost (Laughlin et al, 2009). In addition, the role of the PDE3B gene in the testis is not known. PCTS could be used to challenge testicular tissue with FSH. FSH has been shown to affect phosphodiesterase activity in the testis of rats but the specific function of that activity remains unknown (Conti et al, 1983).

Within the seminiferous tubules of the testes, germ cells undergo a series of divisions ultimately leading to the production of spermatozoa. These processes are collectively termed spermatogenesis, and are regulated by endocrine, paracrine and autocrine communications within the testes. Therefore, a series of experiments were conducted using the stallion as a model to provide a better understanding of the specific gene products involved in spermatogenesis and useful methods to investigate their functions. Ultimately, this research could lead to the development of new strategies to treat and/or prevent conditions that cause testicular dysfunction. The studies discussed here provide new insight into the processes involved in spermatogenesis and novel tools

for investigating testicular function. In addition to our studies in stallion infertility, many of the methods developed here can lead to new research approaches in other species including humans. The ability to accurately identify aberrant molecular mechanisms that cause reproductive inadequacies may increase the number and efficacy of current treatments available to all clinicians and researchers.

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APPENDIX A

Table A. Gene products preferentially expressed in stallion testis tissue in both the light vs. dark microarray experiment (Study 1A) and the fertile vs. subfertile microarray experiment (Study 1B).

	Microarray Study 1A		Microarray Study 1B	
Gene Products	Light Parenchyma	Dark Parenchyma	Fertile Testis	Subfertile Testis
GNG3	-2.0		-1.8	
SERPINF1	-1.8		-2.1	
IFI6	-2.3		-2.0	
CITED1	-2.2		-2.0	
DHCR24		2.5	-2.9	
GSTO1		2.0	-1.8	
GLG1		2.5	-2.9	
SC4MOL		1.6	-1.7	
CDC2	-2.3			1.7

Gene products are listed according to accepted abbreviations. Numbers represent balanced difference values for each gene product.

APPENDIX B

Table B. Mean values of gonadal characteristics and circulating hormone concentrations for ten mature horses.

Testis and Semen Parameters	Individual Stallions									
	1955	75	1906	Arab	1959	1861	1859	1957	Lucky	Peppy
Parenchyma Weight (g)	154.27	199.60	171.85	217.68	243.46	251.64	201.80	209.93	169.54	152.11
DSP (10^6)										
Per gram	15.62	15.91	19.97	16.76	13.40	15.68	14.79	13.17	16.86	13.74
Per testis	2094	3176	3482	3648	3262	3947	2984	2765	2858	2091
Per horse	4680	6703	6566	7270	6421	7918	5492	4552	6450	NA
DSO (10^6)	4580	8534	5507	8142	5323	7714	6253	5358	5791	NA
Apoptotic Rate	15.18	4.56	7.12	6.26	7.14	3.89	5.61	7.14	NA	9.98
T (ng/ml)	1.15	1.84	1.21	2.20	1.17	2.05	1.31	1.46	1.05	0.54
E_2 (pg/ml)	48.89	72.54	40.93	59.98	68.41	66.40	62.55	45.20	28.20	19.27
LH (ng/ml)	13.31	3.63	3.84	8.74	7.13	4.11	6.16	7.18	5.30	5.26
FSH (ng/ml)	3.55	2.18	12.68	2.06	3.37	2.71	2.58	4.57	4.82	3.99
Inhibin (ng/ml)	3.28	5.45	2.52	3.68	6.25	6.50	4.55	2.23	1.63	1.12

APPENDIX C

PROTOCOL FOR *IN SITU* HYBRIDIZATION

A. *In Vitro* Transcription (Riboprobe or Probe Transcription)

1. Place DNA templates (anti-sense gel chunks cut from low-melt gel) at 70°C for 10 minutes, then move to 37°C heat block until ready to use.

2. Add the following reagents to the reaction mix in order at room temperature (RT) or DNA will precipitate.

Reaction mix (can make ½ reactions with 10 µl total volume w/2X ^{35}S)	
DEPC H_2O	3.3 µl
5X transcription buffer	4.0 µl
1 mM DTT	0.2 µl
RNasin	0.8 µl
33 mM rATP + rGTP + rCTP (made by adding equal volumes of the three 100 mM rNTPs)	0.3 µl
100 uM UTP (1:100 dilution of 10 mM rUTP stock)	2.4 µl
DNA template (gel chunk)	4.0 µl
^{35}S-UTP (50 uCi of label, radioactive)	4.0 µl
RNA polymerase (either T7 or SP6 depending on temp.)	1.0 µl
Final Volume	20.0 µl

3. Incubate the reaction at 37°C for 1-2 hours.

4. Conduct probe test

a. after 1 hour, remove 2 µl of transcription reaction, place in new tube with 8 µl of formamide.

b. Heat denature the sample at 70°C for 10 minutes and load samples on an 8M urea, 5% acrylamide short, fat gel (probe test gel). Load 5 µl of dye marker in extra well to evaluate migration.

c. Run probe test gel for 45 minutes at 40 mAmps.

d. Allow transcription reaction to continue for another hour.

e. Expose film wrapped in Saran wrap for 1 hour or overnight.

f. Protect yourself and unexposed film from radiation contamination.

g. Assess probe quality and continue if acceptable.
100 x 10^6 cpm (total) = good probe quality
>60 x 10^6 cpm = fair probe quality
< 60 x 10^6 cpm = poor probe quality (discard)

h. Add 1 µl of RQ1 DNase and 1 µl RNasin to each transcription reaction. Vortex and incubate at 37°C for 15 minutes.

i. Store probes at -80°C overnight if necessary.

5. Clean Probes

a. Develop film and proceed with probe cleanup if all probes are acceptable.

b. Add 30 µl of sterile H_2O to each probe (bring total volume up to 50 µl).

c. Add 50 µl phenol chloroform isoamyl (PCI) (pH 5) to probes, vortex on high for 1 minute, and centrifuge at 10,000 rpm for 4 minutes.

d. Remove upper aqueous layer and add 50 µl of chloroform isoamyl (CI) to tubes, vortex on high for 1 minute and centrifuge at 10,00 rpm for 4 minutes.

e. Remove upper aqueous layer and place in Roche RNA MiniQuick Spin Columns (Roche, Switzerland). Follow procedure outlined by Roche protocol to resuspend columns and spin samples.

f. Determine probe counts by adding 1 µl of cleaned probe to 5 ml of scintillation fluid. Use program 5 on Beckman counter.

g. Heat probes at 68°C for 15 minutes before using as probe for hybridization.

B. Day 1 Slide Preparation

1. Remove slides from -20°C freezer and let sit until RT.

2. Tissue on slides have been embedded in paraffin, therefore, must deparaffinize sections by dipping slides through the following steps.

CitriSolv™ (Fisher Scientific)	2 min
100% Ethanol (ETOH)	2 min
100% ETOH	2 min
95% ETOH	2 min
70% ETOH	2 min
50% ETOH	2 min

3. Next, sections are fixed in 4% paraformaldehyde (pH 7.4) at 4°C for 10 minutes. Use 250 ml per slide rack.

4% paraformaldehyde (to make 500 ml)
Work in fume hood. Heat 100 ml nanopure H_2O to 60°C.
Add to beaker 20 g of paraformaldehyde
1.25 g NaOH pellets
50 ml of 10X PBS
Bring volume up to 500 ml with nanopure water and stir.
Wait to pH until solution clears (approximately 10 min).

4. Wash slides in 0.5X SSC for 5 minutes at RT.

0.5X SSC
25 ml 20X SSC
975 ml DEPC H_2O

5. Wash slides in Proteinase K (20 µg/ml in RNase buffer) for 10 minutes at RT.

6. Wash slides in 0.5X SSC for 5 minutes at RT.

7. Preheat hybridization buffer to 55°C.

Prehybridization and Hybridization Buffer (for 50 ml)
25 ml formamide
6 ml 2.5 M NaCl (5 M = 3 ml)
1 ml 1 M Tris-pH8
0.5 ml 0.5 M EDTA
1 ml 0.5 M sodium phosphate (500 µl at conc. Of 10 mM)
5 g Dextran sulfate
1 ml 50X Denhardt's
5 ml DEPC H_2O
Difficult to dissolve Dextran sulfate. Incubate on a rocker at 37°C for 4 hours. Immediately prior to use, add 1/10 volume of 1M DTT.

8. On the slides, outline (circle) each section with rubber cement to form a reservoir for the addition of probe.

9. Dry slides by blotting and place in a hybridization chamber. Soak a paper towel in box buffer and place in bottom of container.

10. For large sections such as two equine testis sections per slide you need 50 µl of probe per section. To determine how much probe solution to place on slides:

First, determine how many total section you will be covering per probe. (in this example we will use 1 section).
Add 1mM DTT (a total of 10% of overall probe solution to a 50 ml disposable tube. In this case it would be 5 µl. DDT must be made fresh.
Add 2 µl of yeast tRNA for each section to be covered to 50 ml tube.. In this case it would be 2 µl.
Determine the amount of probe to use by the following equation:

500,000/previously determined cpm = X

Add X value to 50 ml tube.
Bring up total volume to 50 µl with Hybridization buffer.

11. Heat probe solution at 70°C for 5 minutes and then place on ice until ready to use.

12. Cover sections with probe solution. Take care in completely covering sections. Use the bottom side of a pipette tip to spread solution over each section. Do not mix solutions among adjacent sections.

13. Cover humidity chamber with slides inside and incubate overnight at 55°C in hybridization oven.

C. Day 2 Slide Washing

1. Heat a shaking water bath to 55°C. Make 2 liters of 0.1 SSC, 10 mM 2-mercaptoethanol (βME), 1 mM EDTA buffer and warm in water bath.

2 L of 0.1 SSC, 10 mM βME, 1 mM EDTA
10 ml 20X SSC
154 µl 13M βME
4 ml 0.5 M EDTA
Bring up to 2000 ml with d H_2O

2. Place slides in a rack and wash as follows (the waste is radioactive and must be disposed of properly).

1. 2X SSC with 10 mM βME, 1 mM EDTA, 10 minutes at RT.

2. Repeat step 1.

3. Add 400 µl of 10 ng/ml RNaseA (previously boiled) to 200 ml of RNase buffer. Dip slides in rack for 30 minutes at RT. Following this wash, waste is not considered radioactive.

4. 2X SSC with 10 mM βME, 1 mM EDTA, 200 ml, 10 minutes, RT.

5. Repeat step 4.

6. Place slides in a large container and cover with 0.1X SSC with 10 mM βME, 1 mM EDTA (approximately 1 liter). Place container with slides into a gently shaking water bath for a minimum of 1 hour.

7. Repeat step 6.

8. Wash in 250 ml of 0.5X SSC for 10 minutes at RT.

9. Repeat step 8.

3. Dehydrate slides in the following ethanol washes:
50% ETOH + 0.3 M NH_4A_c, 2 minutes, RT
70% ETOH + 0.3 M NH_4A_c, 2 minutes, RT
90% ETOH + 0.3 M NH_4A_c, 2 minutes, RT

4. Remove rubber cement by lifting the edge with needle.

5. Blot slides and dry in racks on a tray for 3 hours at 37°C.

6. Line up slides in order on a film cassette. Tape down slides on edges to cassette insert. Expose slides to film at RT. Time of exposure depends on quality of probes used. If probes are above 100,000 cpm, then expose over night to assess quality. If less than 100,000 cpm, expose for 2 -3 days. Make sure to wrap whole cassette in foil in the darkroom.

D. Day 3 Dipping of Slides

1. If autoradiograph is acceptable, the slides are dipped in Kodak NTB2 nuclear emulsion in complete darkness.

Items needed for Emulsion, Dipping, and Developing Slides in Dark Room
42° water bath
Emulsion (Kodak NTB2). When emulsion is received, aliquot into 5 ml containers. When ready to use for slides, use 2 aliquots plus 5 ml of d H_2O.
113.4 ml d H_2O
500 ml flasks
50 ml tubes
Empty slide boxes to place freshly dipped slides for storage and development
Aluminum foil
Dryrite to reduce moisture in slide boxes
Kimwipes
Developer, Fixer, d H_2O
15°C bath, ice bucket and thermometer
3 two liter beakers for rinsing

2. Emulsion is diluted 2:1 with water. Emulsion needs to be at 42°C and poured carefully into a dipmizer without bubbles.

3. Dip and develop a test slide to evaluate emulsion quality. Temperature of developer, water and fixer should be at 42°C (develop for 5 minutes, water 30 seconds, fixer 5 minutes, running water for 5 minutes).

4. Dip experimental slides in emulsion at 42°C two times. Carefully clean back of slides of residual emulsion. Set slides at a diagonal, to allow for excess emulsion to slowly drain off glass, allowing only ends of slides to touch surfaces. Do not lay slides flat. Allow 15 minutes to expire and rotate slides 180° (this step keeps emulsion from pooling on ends which makes for uneven surfaces when trying to apply cover slip). Transfer slides to a slide box with dryrite wrapped in kimwipe placed in corner of box.

5. Place slide box with slides into bread box without lid for 2 hours. This step minimizes dust accumulation on slides and allows them to dry completely.

6. Place lid on slide boxes, tape edges, and completely wrap boxes in foil. Label boxes carefully. Store boxes at 4°C until time to develop.

E. Developing of Slides

1. Remove slides and allow to warm to RT.

2. Develop and stain using the following protocol:

Kodak Developer, 10 minutes, dilute 1:1 with water at 15°C in dark
Distilled Water, 30 seconds
Fixer with Hardener, 15 minutes. Mix carefully.
Water, 5 minutes, 2 times, under running water, can turn on light after this step, slides must remain wet at this point
1% Toluidine Blue, 3 minutes
Water, 45 seconds, 2 times
50% EtOH, 3 minutes
70% EtOH, 2 minutes
95% EtOH, 2 minutes, 2 times

3. Use permount to cover slip slides. Place one drop of xylene and one drop of permount on each section. Carefully lay coverslip onto slide. Remove bubbles by gently pushing down with pipette tip.

F. Recipes for *in situ* hybridization solutions

1. 20X SSC
   173 g Sodium Chloride
   88.2 g Sodium Citrate
   Bring up to 1 liter with distilled water. Add 1 ml Diethyl Pyrocarbonate (DEPC) and shake vigorously. Place at 37°C overnight. Autoclave for 40 minutes.

2. 0.5X SSC
   25 ml 20X SSC
   975 ml DEPC water

3. 2X SSC with 10 mM βME + mM EDTA
   100 ml 20X SSC
   77 µl 13 M βME
   2 ml 0.5 M EDTA
   Bring up to 1 liter with DEPC water

4. 0.1X SSC with 10 mM βME + mM EDTA
   10 ml 20X SSC
   154 µl 13 M βME
   4 ml 0.5 M EDTA
   Bring up to 2 liters with DEPC water

5. RNase Buffer (500 mM NaCl, 10 mM Tris) pH 8.0
   50 ml 5 M NaCl
   5 ml 1 M Tris, pH 8.0
   445 ml Nanopure water

6. Box Buffer (4X SSC + 50% formamide)
   100 ml 20X SSC
   250 ml formamide
   150 ml DEPC water

7. Hybridization Buffer
   25 ml formamide
   6 ml 2.5 M NaCl (5 M = 3 ml)
   1 ml 1 M Tris-pH8
   0.5 ml 0.5 M EDTA
   1 ml 0.5 M sodium phosphate (500 µl at conc. Of 10 mM)
   5 g Dextran sulfate
   1 ml 50X Denhardt's
   5 ml DEPC H_2O
   Difficult to dissolve Dextran sulfate. Incubate on a rocker at 37°C for 4 hours. Immediately prior to use, add 1/10 volume of 1M DTT.

8. 50X Denhardt's (to 500 ml with DEPC water)
   5 g Ficoll 400
   5 g Polyvinylpyrrolidone
   5 g BSA (Pentax Fraction V)

APPENDIX D

RADIOIMMUNOASSAY PROCEDURE FOR MEASURING MEDIA CONCENTRATION OF TESTOSTERONE FOLLOWING PRECISION-CUT *IN VITRO* CULTURE

A. Prepare PBSG

1 L of PBSG	
NaCl	8.170 g
$NaH_2PO_4H_2O$	0.856 g
Na_2HPO_4	0.540 g
EDTA	3.720 g
Thimerosal Powder	0.100 g

1. Dissolve above ingredients in 1000 ml beaker with double distilled H_2O (approximately 900 ml).
2. Measure pH and adjust to 7.4.
3. Add 1.0 g of gelatin (Knox) to EDTA-PBS solution and stir on "low" heat for 2 h to dissolve gelatin.
4. Let PBSG cool to RT before use (store at 4°C).

B. Prepare Charcoal Suspension

500 ml of Charcoal Dextran Suspension	
Charcoal Norit SPXX	3.1250 g
Dextran Pharmacia T-70	0.3125 g
PBSG	600 ml

1. Use fresh charcoal suspension within two weeks old.
2. Stir charcoal suspension for 6 h prior to use.
3. In a 1000 ml beaker, combine charcoal, dextran, and PBSG.
4. Stir to mix at 4°C for 30 m prior to each use..

C. Assay Methodology

1. Ether Extraction of Media Samples
    a. Extractions are conducted in duplicate
    b. Label 16 x 125 mm borosilicate glass disposable culture tubes (VWR Scientific Inc. [KIMAX®51] and additional set of 12 x 75 mm glass tubes to match large tubes.
    c. Pipet 500 µl of unknown sample into appropriately labeled 16 x 125 glass tubes.

d. Under a fume hood, add 5 ml of anhydrous ether (EM Science, Cincinnati, OH) to each 16 x 125 mm tube with unknown.
e. Vortex 4 tubes by hand for 1 min.
f. Place up to 4 tubes at a time into liquid nitrogen (put liquid nitrogen into Styrofoam ice chest with rack to hold tubes) for 10-15 s until media freezes (bottom layer only).
g. Remove tubes from liquid nitrogen and decant liquid into appropriately labeled 12 x 75 mm glass tube.
h. Place 12 x 75 mm glass tubes into nitrous gas dryer (Multivap Analytical Evaporator, Organomation, South Berlin, MA) containing 37°C distilled H_2O until tubes are dry.
i. Reconstitute by adding 500 µl of PBSG to each dried tube and vortex.
j. Samples can be stored at 4°C if not used immediately.

D. Setting up the Assay

1. Label three tubes T, N, 0 and pipet 800 µl, 600 µl, and 500 µl PBSG in duplicate into each tube, respectively.
2. Pipet 500 µl of standard into appropriately labeled tubes, in duplicate. Standards should consist of a total of 12 tubes ranging from 3.9 pg/tube to 8,000 pg/tube.

<u>If new standards are needed:</u>
Take 320 µl of Testosterone (stock) and add 19.68 ml of medium (DMEM with FBS). This equals 8,000 pg/500 µl of standard. Make dilutions by taking 10 ml of 8,000 standard and add to 10 ml of medium. This makes 4,000 pg/500 µl of standard. Continue until reach 3.9 pg/500 µl.

3. Add 100 µl of testosterone antibody (Colorado State University) to each tube except for T and N tubes.
4. Add 100 µl of ^{3}H-testosterone tracer (Perkin Elmer-NEN, Boston, MA) to every tube.
5. Mix the tubes by shaking, covered with foil. Incubate at 4°C overnight.
6. Add 200 µl of charcoal solution to each tube except for the T tube (in cold room).
7. Vortex and incubate at 4°C for 15 minutes.
8. Centrifuge samples for 20 minutes at 2800 rpm (2282 x g).
9. Transfer samples into scintillation tubes containing 5 ml of Ecolume biodegradable scintillation cocktail (MP Biomedicals, Irvine, CA). Cap tubes and shake gently.
10. Count the samples for 1 minute with Beckman Beta counter.
11. Use AssayZap program to calculate concentrations of unknowns in comparison to a known standard curve.

APPENDIX E

RADIOIMMUNOASSAY PROCEDURE FOR MEASURING MEDIA CONCENTRATION OF ESTRADIOL FOLLOWING PRECISION-CUT *IN VITRO* CULTURE

Followed protocol for Diagnostic Systems Lab, Inc. Ultra-Sensitive Estradiol RIA Kit (DSL – 4800) with the following changes:

1. Begin with double extracted samples and use 100 µl of unknown, reconstituted with 500 µl of PBSG. All samples run in duplicate.
2. Allow all reagents to reach RT (~25°C) and mix liquid reagents by inversion.
3. Label and arrange tubes in duplicate for Total Counts, Non-Specific Binding (NBS), Standards, Controls, and unknowns.
4. Add 200 µl of standards, controls or unknowns to appropriate tubes. To NSB tubes, add 300 µl of the 0 pg/ml estradiol standard.
5. Add 100 µl of estradiol antiserum to al tubes except NSB and total count tubes.
6. Vortex all tubes, cover and incubate at RT for 1 hour.
7. Add 100 µl of estradiol [I-125] reagent to each tube.
8. Vortex all tubes, cover and incubate at RT for 2 hours.
9. Add 1 ml of precipitating reagent to all tubes except total count tube. Vortex and allow to stand at RT for 20 minutes.
10. Centrifuge all tubes, excepts total count tubes, for 20 minutes at 3,000 rpm, 4°C.
11. Decant and count in gamma counter for 1 minute.

APPENDIX F

HEAT INACTIVATED, CHARCOAL STRIPPED PROTOCOL FOR REMOVING EXOGENOUS STEROIDS FROM SERUM

Recipe: Dextran-coated charcoal solution (DCC; 10X)

100 ml water
5 g charcoal (Sigma)
0.5 g dextran (70,000 MW)
8.2 g NaCl

1. Add 250 µl of 10X DCC solution for each 50 ml of fetal bovine serum (FBS).
2. Mix by inverting and place in a 56°C shaking water bath for 30 minutes. Invert tubes every 5-10 minutes.
3. Spin tubes at 3000 rpm for 10 minutes.
4. Add 250 µl of DCC to another set of centrifuge tubes.
5. Decant FBS into new tubes.
6. Mix by inverting and place in a 37°C shaking water bath for 30 minutes. Invert tubes every 5-10 minutes.
7. Spin tubes at 3000 rpm for 10 minutes.
8. Decant serum into a clean container.
9. Filter sterilize into a sterile bottle (0.22 µm filter)
10. Aliquot stripped serum and store in freezer.

If FBS has previously been heat inactivated:

1. Add 9.375 g of charcoal + 0.9375 g dextran to 100 ml of FBS.
2. Stir to mix well for 10 minutes.
3. Pour into four 50 ml tubes (25 ml each for total of 4 tubes) and spin at 2,000 rpm for 2 hours.
4. Decant serum into clean containers and filter through 500 ml filter (Corning 25943-500; 0.45 µm cellulose acetate low protein binding membrane.
5. Aliquot and store in freezer.

APPENDIX G

PROTOCOL FOR LACTATE DEHYDROGENASE (LDH) ASSAY

1. Add 25 ml of tissue culture grade water to lyophilized LDH enzyme.

2. Aliquot out enzyme after initial use, do not want to continually freeze and thaw.

3. For assay, you want a total volume of 120 µl per well of medium (50% total assay mixture and 50% culture medium). Need equal amounts of LDH assay substrate, enzyme and dye solution; therefore, added 20 µl of each for a volume of 60 µl.

4. Prepare mixture at time of use and add 120 µl to wells of a 96 well plate, in triplicate.

5. Cover plate with foil and put in dark (away from light exposure) for 25 minutes.

6. After incubation, add 1/10 volume (18 µl) of 1N HCl to terminate reaction.

7. Read plates at 490 nm and then at 690 nm. Subtract the 690 nm reading from the 490 nm reading. Read plates on Packard BS10000 Spectracount microplate reader.

APPENDIX H

PROTOCOL FOR USE OF ZEISS AXIOPLAN 2 MICROSCOPE

A. Brightfield and Darkfield image capture
    1. Turn general power on.
    2. Turn on computer.
    3. Turn on microscope and make sure green light on camera is on before proceeding.
    4. Open Axiovision, click on "microscope" (top menu), then click on 50:50 for photo and eye view on computer.
    5. Set Kohlor Illumination
        a. Select objective.
        b. Focus on slide with computer (live view and then scope view).
        c. Focus each eyepiece individually and then together.
        d. Find and close field diaphragm, (right base of scope).
        e. Sharpen edges by moving condenser up and down.
        f. Center field of diaphragm with little screws under stage.
        g. Expand field of diaphragm to be just out of view, may need to sharpen edges again.
        h. Close diaphragm above condenser (silver ring above DIC).
        i. Remove left eyepiece and adjust diaphragm so you can just see all edges through the scope in the field of view (FOV).
        j. Capture images.
    6. Turn on camera and click measurement
    7. For image capture, use RGB
        a. For 10X, use 1300X1030 resolution or 2550X1920
        b. For 5X use 2600X resolution
        c. For 2.5X use highest resolution 3900X3090
    8. Brightfield image capture
        a. Use any DIC settings.
        b. Can color adjust by clicking "white balance", then "interactive" button (you must then click on image where you think it is white in color).
    9. For Darkfield image capture use "D" settings.
B. Cleaning slides for quality images
    1. Clean slide surfaces only with cotton swabs and either Windex or 100% EtOH.
    2. Do not smear mount medium across coverslip when cleaning surface.
C. Saving images
    1. To save, click "snap" on camera icon in upper left panel.
    2. Can save images as ZVI, TIFF, or JEPG.
    3. Save images as 8 bit.
    4. At this point, discard color, size, crop, adjust contrast and save images.

APPENDIX I

PROTOCOL FOR PRECISION-CUT TISSUE SLICES

I. DAY PRIOR TO EXPERIMENT

1. Clean the slicer, culture inserts and other glassware required for the procedure. Prepare the slicing buffer and culture media.

II. DAY OF THE EXPERIMENT

1. Assemble the slicer. Make sure that the circular blade is in the correct orientation (engraving facing up) and that the size of the tissue core is the same as the insert in the slicer. Begin gassing the slicing buffer (make sure that the buffer is circulating through the slicer and that there are no bubbles in the slicing chamber or tubing) and running the circulating water bath.

2. Harvest the organ(s) of interest and place in slicing buffer on ice. Try to remove as much blood from the organ by gently 'massaging' the tissue and changing buffer.

3. Generate tissue cores by placing the organ on dental wax and carefully 'rotating' the coring tool through the tissue. This step is critical -- poor cores translate into poor slices. The tissue cores are placed into slicing buffer (4 ^{o}C).

4. Place the tissue core into the slicing insert; make sure that core is not sitting at an angle. The tissue core will fit firmly in the corresponding insert. Begin rotation of the blade using the power pack - it is critical that the blade is rotating clockwise and that the slicing insert is moved over the blade in a uniform, precise manner. The speed which the tissue core is moved across the blade depends on both the organ and species used. Despite the availability of a reliable tissue slicer, the production of optimal precision-cut tissue slices is also dependent upon the skill and attention of the investigator.

5. The production of optimal, reproducible slices can be evaluated by i) slice thickness or ii) slice weight. It is advantageous to the investigator to determine slice thickness during the procedure since the viability of slices is highly dependent upon the thickness of the slice.

6. Precision-cut tissue slices are harvested from the slicer into ice-cold slicing buffer. Slices are loaded onto culture inserts (1 to 4 slices per insert). During this step the investigator should handle the slices with care (slices should

only be handled with a loading tool - not forceps). The slices should be entirely on the mesh screen and lying flat. The screens are then blotted and placed into the 20 ml scintillation vials containing 1.70 ml of media. The vials are then capped (the caps have a 1 mm hole in them) and placed into the Vitron incubator which is gassed with 95:5 O_2/CO_2 at a flow rate of 1 ml/min. The incubator should be cleaned with 70% EtOH before the experiment.

It is important to proceed from sacrificing the animal to organ culture of the slices in an expedient fashion, however do not rush at the expense of generating reproducible, optimal precision-cut tissue slices.

7. Pre-incubate tissue slices for 1 - 2 hr prior to the addition of test compounds. This allows the tissue to recover from the slicing process. Precision-cut tissue slices can be cultured for various times, depending on the conditions and organ employed.

8. Following the experiment, the slicer and culture inserts are soaked in Tergazyme overnight and repeatedly rinsed prior to the next experiment.

BUFFERS

KREBS-BICARBONATE SLICING BUFFER

NaCl	27.6 g
KCl	1.44 g
KH_2PO_4	0.52 g
$NaHCO_3$	8.44 g
Glucose	18.0 g
$CaCl_2:2H_2O$	1.70 g
$MgSO_4:7H_2O$	1.18 g
ddH_2O	4 lt

Add the chemicals in order; before adding the calcium chloride and magnesium sulfate dissolve each chemical separately in a small volume of water, then add to the buffer. Refrigerate and bubble with 95:5 $O_2:CO_2$ for 30 min before pH to 7.40, use within 1 week.

SLICING BUFFER

D-gluconic acid, potassium salt	53.5 g
L-ascorbic acid, free acid	8.84 g
D-saccharic acid, monopotassium salt	32.7 g
L-glutamine	1.40 g
Potassium phosphate, monobasic, anhydrous	14.2 g
Potassium citrate monohydrate	2.72 g
Succinic acid, free acid	0.98 g
Magnesium sulfate, heptahydrate	5.15 g
Magnesium chloride, hexahydrate	1.70 g
Potassium bicarbonate	0.84 g
Glucose	1.50 g
Fructose	1.50 g
Adenosine, free base	5.66 g
Glutathione (reduced)	7.75 g
Deferoxamine	13.8 mg
ddH_2O	4 lt

pH to 7.40 with saturated KOH, use within one week.

VITA

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